Struktur und räumliche Verteilung mikrobieller Gemeinschaften im Verdauungstrakt ausgewählter Boden-Invertebraten

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„Jeder dumme Junge kann einen Käfer zertreten,
aber alle Professoren der Welt können keinen herstellen.“

aus China

Meinen Eltern, und meiner lieben Frau Dorit

Die in dieser Dissertation beschriebenen Ergebnisse sind in folgenden Originalpublikationen veröffentlicht bzw. zur Veröffentlichung eingereicht:

1. **Egert, M. und M.W. Friedrich** (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl. Environ. Microbiol*. **69**:2555-2562.
2. **Egert, M., T. Lemke, B. Wagner, A. Brune und M.W. Friedrich** (2003) Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol*. **69**: 6659-6668.
3. **Lemke, T., U. Stingl, M. Egert, M.W. Friedrich und A. Brune** (2003) Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol*. **69**: 6650-6658.
4. **Egert, M., S. Marhan, B. Wagner, S. Scheu und M.W. Friedrich**. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). Zur Veröffentlichung eingereicht bei *FEMS Microbiol. Ecol*.

Folgende Manuskripte werden als Entwürfe präsentiert:

5. **Egert, M., U. Stingl, L. Dyhrberg Bruun, B. Wagner, A. Brune und M.W. Friedrich**. Physicochemical gradients and microbial diversity in the intestinal tract of the phytophagous larva of *Melolontha melolontha* (Coleoptera: Scarabaeidae). Einreichung geplant bei *Appl. Environ. Microbiol*.
6. **Egert, M., L. Dyhrberg Bruun, B. Wagner, A. Brune und M.W. Friedrich**. Analysis of microbial community structure and topology in the intestinal tract of the larva of *Melolontha melolontha* (Coleoptera: Scarabaeidae) reveals an enrichment of *Desulfovibrio*-related bacteria at the hindgut wall. Einreichung geplant bei *Appl. Environ. Microbiol*.

Inhaltsverzeichnis

Abkürzungen

apsA:	Gen für die α-Untereinheit der Adenosin-5´-Phosphosulfat-Reduktase
CFB:	*Cytophaga-Flavobacterium-Bacteroides*
CLSM:	Konfokales Laser-Scanning-Mikroskop
DAPI:	4´,6-Diamidino-2-phenylindol
DFA:	discriminant function analysis
DGGE:	Denaturierende-Gradienten-Gel-Elektrophorese
FAM:	6´-Carboxyfluorescein
FG:	Frischgewicht
FISH:	Fluoreszenz in-situ Hybridisierung
ITS:	internal transcribed spacer
L1-3:	Entwicklungsstadien der Scarabaeidenlarven
MDS:	multi-dimensional scaling
OTU:	operative taxonomische Einheit
PCR:	Polymerase Kettenreaktion
PBS:	phosphate buffered saline
PFA:	Paraformaldehyd
RT:	reverse Transkriptase
T-RF:	terminales Restriktionsfragment
T-RFLP:	terminaler Restriktionsfragment-Längen-Polymorphismus

Alle weiteren Abkürzungen wurden entsprechend der „Information for authors“ des *European Journal of Biochemistry* verwendet.

Zusammenfassung

Boden-Makroinvertebraten sind entscheidend an der Transformation organischer Substanz beteiligt, die für viele Schlüsselfunktionen des Bodens verantwortlich ist. An den Transformationsprozessen, die während der Darmpassage ingestierter organischer Substanz ablaufen, sind intestinale Mikroorganismen beteiligt, die auch für die Ernährung ihrer Wirte von entscheidender Bedeutung sind. Über die Zusammensetzung der mikrobiellen Gemeinschaften im Verdauungstrakt der meisten Boden-Invertebraten sowie ihre räumliche Verteilung innerhalb verschiedener Darmabschnitte ist allerdings nur wenig bekannt. Gerade die Topologie mikrobieller Gemeinschaften wird aber als eine wichtige Voraussetzung zum tieferen Verständnis ihrer Funktion in Verdauungstrakten von Invertebraten angesehen, die von ausgeprägten axialen und radialen Gradienten physiko-chemischer Parameter geprägt sind.

Im Rahmen dieser Arbeit wurden daher Struktur und räumliche Verteilung mikrobieller Gemeinschaften im Verdauungstrakt der Larven zweier Scarabaeiden (*Pachnoda ephippiata* [Kongo-Rosenkäfer] und *Melolontha melolontha* [Feldmaikäfer]) und von Regenwürmern (*Lumbricus terrestris*) mit Methoden der molekularen mikrobiellen Ökologie untersucht. Während der Untersuchungen wurde zudem ein neuartiger PCR-Artefakt, die Bildung sog. pseudo-T-RFs, mit bedeutenden Auswirkungen für die T-RFLP-Analyse mikrobieller Gemeinschaften entdeckt, beschrieben und mögliche Ansätze zu seiner Vermeidung aufgezeigt.

In einer T-RFLP-Studie mit Regenwürmern wurde gezeigt, dass sich die Darmmikrobiota dieser Tiere aus der Nahrung rekrutiert, d.h. ihnen im Gegensatz zu vielen anderen Boden-Invertebraten eine spezifische Darmflora fehlt. Es wurde deutlich, dass die mit dem gefressenen Boden aufgenommene mikrobielle Gemeinschaft während der Darmpassage signifikante Veränderungen ihrer relativen Zusammensetzung erfährt und dass die Unterschiede zwischen den mikrobiellen Gemeinschaften von Futter, Darm und Losung stark von der Diät der Regenwürmer beeinflusst werden.

Die hier präsentierten Ergebnisse zur Intestinalmikrobiologie von Scarabaeidenlarven stellen die ersten ihrer Art für Käferlarven und mit die ersten für andere Boden-Arthropoden als Termiten dar. Es konnte gezeigt werden, dass sich die ausgeprägten Unterschiede physiko-chemischer Parameter (pH-Wert, Redoxpotential, Fettsäurespektren), die zwischen den Haupt-Darmabschnitten (Mittel- und Enddarm) der Larven herrschen, in einer deutlich unter-

schiedlichen Besiedlung mit Mikroorganismen widerspiegeln. Im Gegensatz zu den untersuchten Regenwürmern ist die Darmmikrobiota der Scarabaeidenlarven als spezifische Darmflora anzusehen, da sie deutlich verschieden zur Mikrobiota der aufgenommenen Nahrung war. Bei beiden Larven war der Mitteldarmabschnitt weniger dicht besiedelt als der als Gärkammer angesehene Enddarm. Die Methanogenese war stets auf den Enddarm beschränkt; bei den Maikäferlarven wurden *Methanobrevibacter*-Arten, bei den Rosenkäferlarven zusätzlich *Methanomicrococcus*-Arten als verantwortliche Methanogene identifiziert. Im Vergleich zu den *Bacteria* war aber sowohl die Diversität als auch die relative Häufigkeit der *Archaea* sehr gering. Die phylogenetische Analyse der Bakteriengemeinschaften zeigte eine sehr große Diversität auf, die offensichtlich viele bislang unkultivierte Arten umfasst. Die überwiegende Mehrheit aller Sequenzen ließ sich den *Actinobacteria*, *Bacillales*, *Bacteroidetes*, *Clostridiales*, *Lactobacillales* und *Proteobacteria* zuordnen; viele Klone gruppierten mit Klonen und Isolaten aus anderen Intestinalsystemen, ein weiterer Beleg für die Darmspezifität der Scarabaeiden-Mikrobiota. Die Verwandtschaft vieler Klone zu hydrolytischen, cellulolytischen und gärenden Isolaten stand in Einklang mit den Fettsäureprofilen der Darmabschnitte (v.a. Acetat und Lactat) und deutet die Beteiligung von Mikroorganismen an der Transformation organischer Substanz nach dem Modell einer anaeroben Nahrungskette zumindest im Enddarm an. Ob dies auch für den Mitteldarmabschnitt gilt, ist noch unklar, da bei den Maikäferlarven keine stabile Darmmikrobiota in diesem Kompartiment nachgewiesen werden konnte.

Am Enddarm der Maikäferlarven wurde erstmals für Arthropoden eine umfassende Analyse der mikrobiellen Gemeinschaften in den Unterfraktionen Wand und Lumen eines Darmabschnittes durchgeführt. Hierbei wurden ausgeprägte Unterschiede in der Besiedlung dieser beiden Fraktionen festgestellt, die als Anpassungen an morphologische (Chitinbäumchen an der Enddarmwand) und eventuell auch physiko-chemische Unterschiede (Gradient eindringenden Sauerstoffs) zwischen Darmwand und -lumen interpretiert werden können. Der auffälligste Unterschied war eine hohe Abundanz (10 - 15% aller Bakterien) *Desulfovibrio*-verwandter Bakterien an der Enddarmwand, die sowohl mit PCR-abhängigen als auch PCR-unabhängigen Methoden abgesichert werden konnte. In seiner Eindeutigkeit ist dieser Befund für Arthropoden bislang einmalig.

1 Einleitung

1.1 Die Bedeutung der Bodenfauna für den Umsatz organischer Substanz im Boden

Art und Menge der toten organischen Substanz eines Bodens („Humus“) beeinflussen nachhaltig seine physikalischen und chemischen Eigenschaften (Gefüge, Nährstoffgehalt und -speichervermögen, Wasser-, Luft- und Wärmehaushalt) und damit seine Fruchtbarkeit (Schachtschabel et al., 1998). Der Umsatz organischer Substanz entscheidet außerdem, ob Böden als Quellen oder Senken für CO_2 und weitere wichtige Spurengase fungieren, die Auswirkungen auf das globale Klima haben (Amundson, 2001; Conrad, 1996). An Bildung, Umsetzung und Abbau der Humusfraktion eines Bodens ist die Bodenfauna in entscheidendem Maße beteiligt (Brussard und Juma, 1996; Lavelle et al., 1995; Lavelle, 2000; Wolters, 1991; Wolters, 2000).

Bodentiere werden anhand verschiedener Merkmale klassifiziert, z.B. nach Größe (Mikro- [< 0,2 mm], Meso- [0,2 - 2,0 mm], Makro- und Megafauna [> 2,0 mm]) (Schachtschabel et al., 1998) oder über Nahrungspräferenzen (z.B. saprophag, microphytophag, zoophag) (Wolters, 2000). Vertreter der Mikrofauna (z.B. Amöben, Ciliaten, Flagellaten) besiedeln Flüssigkeitsfilme um Bodenpartikel, während die Mesofauna (z.B. Fadenwürmer, Springschwänze, Milben) im Porensystem lebt. Allein die Vertreter der Makro- und Megafauna sind in der Lage, einen Boden auch nachhaltig physikalisch zu verändern, v.a. für Regenwürmer, Ameisen und Termiten wurde daher der Begriff „ecosystem-engineers“ geprägt (Jones et al., 1994; Lavelle et al., 1997). Die Möglichkeiten, wie Bodentiere auf die Umsetzung organischer Substanzen im Boden Einfluss nehmen können, sind vielfältig. Als besonders wichtig werden Interaktionen zwischen Bodentieren und Mikroorganismen (Bakterien, Archaea, Pilzen) angesehen, die letztlich für ca. 90% der Mineralisierung im Boden verantwortlich gemacht werden (Lavelle et al., 1995; Lavelle, 2002). Ein wichtiger Ort, an dem solche Interaktionen stattfinden, ist der Intestinaltrakt von saprophagen Makroinvertebraten, die im Ökosystem Boden die Funktion der Primärzersetzer erfüllen (Lavelle, 2002; Scheu, 2002; Wolters, 2001).

Die Umsetzung organischer Substanz beginnt bereits mit der mechanischen Zerkleinerung der Nahrung durch die Mundwerkzeuge der Tiere, da sich hierdurch die angreifbare Oberfläche stark vergrößert. An den Umsetzungsprozessen im Intestinaltrakt sind zum einen die Tiere

selber (z.B. über die Ausscheidung von Verdauungsenzymen) als auch Mikroorganismen beteiligt. Diese Mikroorganismen können mit der Nahrung aufgenommen worden sein oder als Symbionten bzw. Kommensalen in den Tieren leben. Durch die Bewegungen des Intestinaltraktes werden Mikroorganismen und organische Substanz in engsten Kontakt miteinander gebracht, wodurch Umsetzungsprozesse stimuliert werden. Dabei kann es zu einem Abbau organischer Substanz kommen, wenn Polymere pflanzlichen oder tierischen Ursprungs zu Oligo- oder Monomeren hydrolysiert und von Mikroorganismen zu Gärprodukten umgesetzt werden, von denen sich der Wirt letztlich ernährt (s. Kapitel 1.2). Andererseits kann sich die Stabilität organischer Substanz während der Darmpassage auch erhöhen, indem z.B. durch die innige Vermischung mineralischer und organischer Bodenbestandteile stabile Ton-Humus-Komplexe entstehen, in denen die organische Substanz vor weiterem Abbau geschützt ist (Wagner und Wolf, 1998). Abbau und Stabilisierung organischer Substanz sind im Invertebratendarm daher als untrennbar verbundene Prozesse anzusehen (Wolters, 2000).
Die meisten Interaktionen von Boden-Invertebraten mit Mikroorganismen beim Umsatz organischer Substanz im Boden sind noch unverstanden. Obwohl die Verdauungstrakte vieler Boden-Invertebraten dicht mit Mikroorganismen besetzt sind (s. Kapitel 1.2 und 1.3), ist sowohl deren Herkunft (mit der Nahrung aufgenommen oder spezifisch im Darm lebend) als auch ihre Funktion bei der Transformation organischer Substanz und für die Ernährung des Wirtes (Nahrung, Symbionten, Kommensalen, Parasiten) oftmals nicht bekannt. Unter den Bodentieren stellen Termiten die Invertebraten dar, die mit Abstand am Besten auf ihre Interaktionen mit intestinalen Mikroorganismen untersucht sind.

1.2 Transformation organischer Substanz im Termitendarm

Termiten (*Isoptera*) besiedeln ca. 2/3 der Landoberfläche zwischen dem 48° nördlicher und dem 45° südlicher Breite. In tropischen und subtropischen Regionen werden stellenweise mehr als 6000 Individuen m^{-2} gezählt, wobei ihre Biomasse mit Werten von 5 - 50 g m^{-2} die von herbivoren Säugetieren deutlich übersteigen kann (Collins, 1989; Lee und Wood, 1971). Allein die hohen Biomassezahlen legen nahe, dass Termiten entscheidend am Umbau organischer Substanz sowie dem C- und N-Umsatz im Boden ihrer Verbreitungsgebiete beteiligt sind (Brussard und Juma, 1996; Martius, 1994; Wolters, 2000; Wood, 1988). Ihr Beitrag zur jährlichen, globalen Methanproduktion wird auf 1,5 - 7,4 Tg a^{-1} (Sugimoto et al., 1998) bzw.

19,7 ± 1,5 Tg a^{-1} geschätzt (Sanderson, 1996), dies entspricht immerhin einem Anteil zwischen 0,3 und 4%. Während die phylogenetisch niederen Termiten ausnahmslos xylophag sind (Holz und Gras fressend) findet man bei den höheren Termiten (*Termitidae*) neben Pilzkultivierenden v.a. auch humivore Arten (Abe et al., 2000). Humivore Termiten können bis zu 45 t Boden ha^{-1} a^{-1} umsetzen (Bignell, 1994), im Gegensatz zur Xylophagie ist diese Art der Ernährung aber deutlich weniger gut untersucht (Brauman et al., 2000). Der Prozess der Verdauung von Lignocellulose, dem Hauptnahrungsbestandteil xylophager Termiten, ist ein komplexer Prozess, an dem die Termite selber (mechanische Zerkleinerung der Nahrung, Sekretion von Verdauungsenzymen, z.B. Cellulasen) als auch symbiontische Mikroorganismen im Darm (Bakterien, Archaea, Pilze und – bei niederen Termiten – cellulolytische Protozoen) beteiligt sind (Breznak und Brune, 1994; Brune, 2003; Ohkuma, 2003) (Abb. 1.1 A und B).

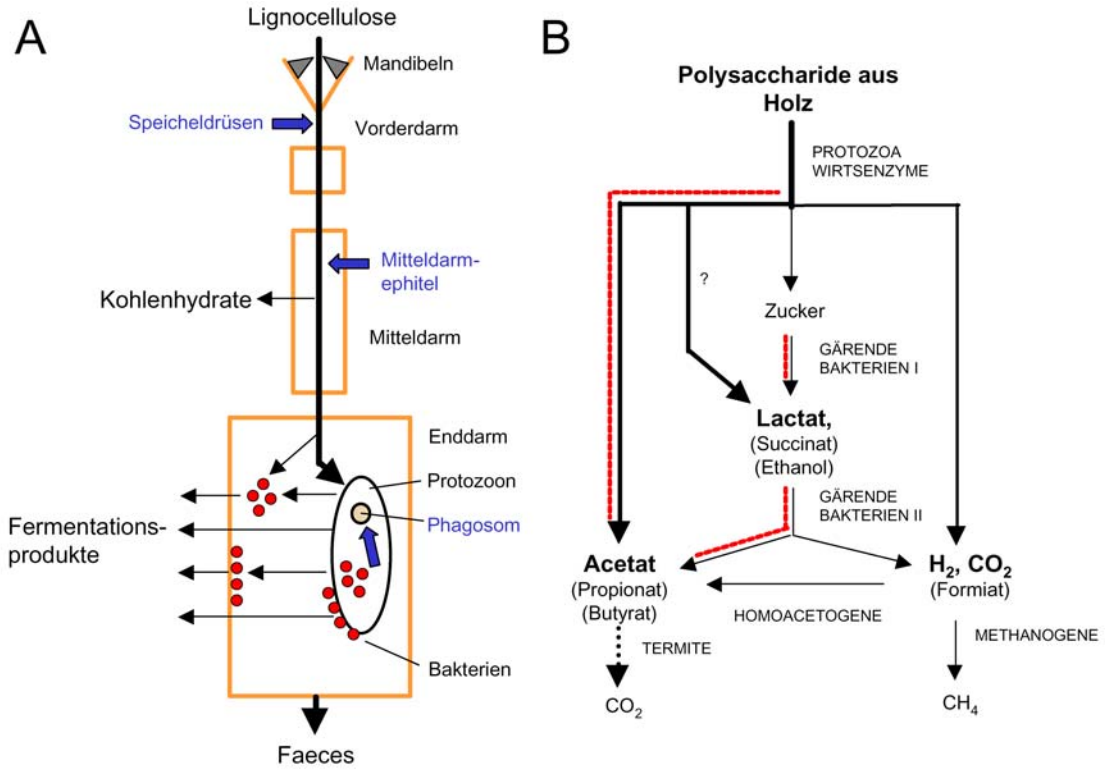

Abbildung 1.1: (A) Symbiotische Verdauung von Lignocellulose durch Holz fressende Termiten. Fette Linien zeigen den Weg unlöslicher Stoffe, dünne Linien den Weg löslicher Abbauprodukte, blaue Pfeile auf Sekretionsorte cellulolytischer Enzyme. Bakterien können als Endobionten oder Epibionten der Protozoen, frei im Darmlumen oder an der Darmwand vorkommen. (B) Schematische Darstellung wichtiger metabolischer Prozesse beim fermentativen Abbau von Polysacchariden im Termiten-Enddarm, basierend auf Ergebnissen erzielt mit *Reticulitermes flavipes*. Rot gepunktete Linien deuten metabolische Flüsse an, die durch den kontinuierlichen Sauerstoffeinstrom durch die Enddarmwand stark beeinflusst werden (beide Abbildungen verändert nach (Brune, 2003)).

Aufgrund ihrer geringen Größe und der Tatsache, dass über die Tracheen herangeführter Sauerstoff bis zu 200 µm weit in das Darmlumen eindringen kann, sind Termitendärme nur partiell anaerob (Brune, 1998). Dies hat zum einen Auswirkungen auf die Zusammensetzung der mikrobiellen Gemeinschaften im Darm, die auch aerobe Mikroorganismen umfasst, als auch auf die dort ablaufenden metabolischen Prozesse (Brune, 1998; Brune und Friedrich, 2000). Tholen und Brune (Tholen et al., 1997; Tholen und Brune, 2000) konnten z.B. zeigen, dass mikrobielle Reduktion des eindringenden Sauerstoffs durch Milchsäurebakterien zu einer Verschiebung des Produktspektrums weg vom Lactat hin zu mehr Acetat führt (Abb. 1.1 B). Neben ihrer Beteiligung am Polymerabbau stellen die Darmmikroorganismen auch eine wichtige Stickstoffquelle für ihre Wirte dar, die sich von sehr stickstoffarmer Kost ernähren (C:N-Verhältnis in Holz bis 1000:1, (Nardi et al., 2002)). In den Enddarm ausgeschiedenen Endprodukte des Stickstoffwechsels (z.B. Harnstoff oder Harnsäure) werden von der Darmflora mineralisiert, assimiliert und der Termite in Form von mikrobieller Biomasse durch proctodeale Trophallaxis wieder zur Verfügung gestellt (Brune, 2003). Außerdem sind viele Darmmikroorganismen in der Lage, molekularen Luft-Stickstoff zu fixieren und so das Stickstoffbudget ihrer Wirte aufzubessern (Lilburn et al., 2001; Noda et al., 1999; Ohkuma et al., 1999). Die durch Darmbakterien in Arthropoden fixierte Menge an Luftstickstoff kann bis zu 40 kg ha^{-1} a^{-1} betragen (Nardi et al., 2002).

Im Gegensatz zu xylophagen Termiten ist der Verdauungstrakt humivorer Termiten größer, länger und sowohl morphologisch als auch physiko-chemisch stärker strukturiert (Bignell, 1994; Brauman et al., 2000). Der Darm von *Cubitermes*-Arten lässt sich z.B. in 8 verschiedene Abschnitte gliedern, entlang derer der intestinale pH-Wert zwischen 5 und 12 variiert (Brune und Kühl, 1996). Auch die Konzentrationen von Wasserstoff und Sauerstoff zeigen starke Schwankungen entlang der Darmachse (Schmitt-Wagner und Brune, 1999). Kappler und Brune (Kappler und Brune, 1999) konnten zeigen, dass der hohe pH-Wert in den vorderen Enddarm-Abschnitten von *Cubitermes*-Arten im Zusammenspiel mit dem in der Darmperipherie eindringenden Sauerstoff das Ablösen von Huminstoffen aus der Bodenmatrix erleichtert, ihr Molekulargewicht verschiebt und ihre Löslichkeit erhöht, wodurch sich die Zugänglichkeit der organischen Substanz für den enzymatischen Abbau durch Wirtsenzyme und Mikroorganismen erhöht. Fütterungsversuche mit radioaktiv markierten Huminsäuren haben weiterhin gezeigt, dass humivore Termiten v.a. die leichter mineralisierbaren Peptidanteile und kaum die aromatischen Bestandteile von Huminsäuren verwerten (Ji et al., 2000), wie

z.T. vermutet worden war (Bignell, 1994). Weitere Fütterungsversuche legten nahe, dass auch pflanzliche Strukturpolysaccharide und mikrobielle Biomasse wichtige Nahrungsgrundlage für Boden fressende Termiten sind (Ji und Brune, 2001).

Die dichte mikrobielle Besiedlung der Intestinaltrakte Boden fressender Termiten (Bignell et al., 1980) und die hohen Konzentrationen mikrobieller Fermentationsprodukte (v.a. Acetat, (Tholen, 1999)) deuten auf aktive mikrobielle Gemeinschaften in den verschiedenen Darmabschnitten hin, deren Funktion im Einzelnen aber noch völlig unverstanden ist. Angesichts des morphologisch und physiko-chemisch stark differenzierten Verdauungstraktes Boden fressender Termiten und ihrer mittlerweile eindeutig nachgewiesenen Fähigkeit, selber Cellulasen zu bilden (Watanabe und Tokuda, 2001), wurde die Notwendigkeit einer symbiontischen Mikroflora hier sogar generell in Frage gestellt (Bignell, 1994). Jüngste Untersuchungen der mikrobiellen Gemeinschaften im Darm Boden fressender Termiten (Friedrich et al., 2001; Schmitt-Wagner et al., 2003a; Schmitt-Wagner et al., 2003b) haben allerdings große Unterschiede zu den Gemeinschaften im gefressenen Boden aufgezeigt, so dass die Darmflora dieser Termiten nicht einfach als eine unter Darmbedingungen florierende Bodenflora sondern vielmehr als eine spezifische Darmflora angesehen werden kann. Weiterhin zeigten sich signifikante Unterschiede in der Besiedlung einzelner Darmabschnitte. Dass zu einem tieferen Verständnis der Verdauungsprozesse gerade diese räumliche Verteilung der Mikroorganismen entscheidend ist, haben Versuche von Schmitt-Wagner und Brune gezeigt (Schmitt-Wagner und Brune, 1999). Sie vermuteten auf Grundlage von Versuchen an isolierten *Cubitermes*-Därmen, dass die Methanogenese in hinteren Enddarmbereichen durch einen cross-epithelialen Wasserstofftransport aus vorderen, morphologisch aber eng benachbarten Darmabschnitten getrieben wird. An Schabendärmen konnte ein derartiger Transport direkt nachgewiesen werden (Lemke et al., 2001).

1.3 Scarabaeidenlarven und Regenwürmer als weitere Modellsysteme für die Transformation organischer Substanz

Neben Termiten gehören Asseln, Diplopoden, Dipterenlarven, Käferlarven und insbesondere Regenwürmer zu den wichtigsten Vertretern der saprophagen Makrofauna des Bodens.

Larven von Scarabaeiden (Blatthornkäfern), einer ca. 25.000 Arten umfassenden Käferfamilie (Klausnitzer, 2002), stellen Vertreter verschiedenster Nahrungsgilden (phytophag, coprophag, saprophag, humiphag) dar. Sie bieten sich als Modellorganismen zum Studium intestinaler

Transformationsprozesse an, da ihr Verdauungssystem auffallende Parallelen zum gut untersuchten Termitensystem aufweist. Bereits frühe Untersuchungen an *Melolontha melolontha* (Feldmaikäfer), *Oryctes nasicornis* (Nashornkäfer), *Cotnis nitida* (Junikäfer) und *Osmoderma scabra* zeigten, dass die Gliederung des Verdauungstraktes in einen z.T. stark alkalischen, mit hydrolytischen Enzymaktivitäten ausgestatteten Mitteldarm sowie einen eher neutralen, von mikrobiellen Fermentationsprozessen gekennzeichneten Enddarm über die gesamte Familie der Scarabaeiden verbreitet ist (Grayson, 1958; Rössler, 1961; Wiedemann, 1930). Viele Larven können allein von totem organischen Material (Torf) leben, Larven von *Adoryphorus couloni* produzieren dabei ihr eigenes Körpergewicht an Faeces pro Tag (McQuillan und Webb, 1994). Genauere Untersuchungen zu den Transformationsprozessen im Darm gibt es allerdings nur bei *Oryctes nasicornis* (Bayon, 1980; Bayon und Mathelin, 1980) und *Pachnoda marginata* (Cazemier et al., 1997b). Wie Termitendärme sind die Intestinaltrakte von Scarabaeidenlarven dicht mit Mikroorganismen besiedelt (Cazemier et al., 1997a), deren Bedeutung für die Verdauung auch schon früh erkannt wurde (Werner, 1926), über deren Zusammensetzung oder gar Funktion allerdings kaum etwas bekannt ist. Bei der Mehrzahl der aus Scarabaeidenlarven bislang isolierten Bakterien handelt es sich um potentiell entomopathogene Mikroorganismen (z.B. *Serratia* spp. oder *Paenibacillus* spp.). Dies erklärt sich damit, dass ein Schwerpunkt mikrobiologischer Arbeiten mit Scarabaeidenlarven, die viele bedeutende Pflanzenschädlinge umfassen, die Suche nach Mikroorganismen mit potentieller Eignung für die biologische Schädlingsbekämpfung ist (Klein und Jackson, 1992). Eine Ausnahme ist *Promicromonospora pachnodae*, ein hemicellulolytisches Actinobacterium, das aus dem Enddarm humivorer *Pachnoda marginata* Larven isoliert wurde (Cazemier et al., 2003).

Die überragende Bedeutung von Regenwürmern (*Oligochaeta : Lumbricidae*) für die physikalischen Eigenschaften von Böden, den Streuab- und umbau und damit den C- und N-Haushalt in Böden ist unbestritten (Edwards, 1998) und wurden bereits Ende des 19. Jahrhunderts von Charles Darwin erkannt und beschrieben (Darwin, 1881). Viele der positiven Einflüsse von Regenwürmern auf die Eigenschaften eines Boden werden auf Interaktionen mit Mikroorganismen, auch während der Darmpassage zurückgeführt (Doube und Brown, 1998; Edwards und Fletcher, 1988). Allerdings gilt auch hier, dass diese Interaktionen bislang kaum verstanden und v.a. mit molekularen, kultivierungsunabhängigen Methoden (s. Kapitel 1.3) nur wenig untersucht sind. Im Allgemeinen wird angenommen, dass Mikroorganismen (neben organischem Material wie z.B. Dung oder Blättern) für Regenwürmer v.a. Nahrung darstellen, wobei für Bakterien eine wesentlich geringere Bedeutung angenommen wird als

wobei für Bakterien eine wesentlich geringere Bedeutung angenommen wird als für Algen, Protozoen und insbesondere Pilze (Edwards und Bohlen, 1996; Edwards und Fletcher, 1988). Im Gegensatz zu anderen Boden-Invertebraten gibt es bislang kaum Hinweise, dass Regenwürmer eine (abundante) spezifische Darmflora besitzen (Jolly et al., 1993; Toyota und Kimura, 2000). Während v.a. frühere Studien zeigten, dass die Flora im Darm von Regenwürmern der des gefressenen Bodens sehr ähnlich ist (Bassalik, 1913; Parle, 1963), konnten spätere Arbeiten aber doch Unterschiede in ausgewählten phylogenetischen Gruppen (z.B. *Proteobacteria*, (Schönholzer et al., 2002)) oder funktionellen Gilden (z.B. Cellobiose-Verwertern, (Karsten und Drake, 1995)) aufzeigen. Für endogäische, tropische Regenwürmer wurde sogar die Hypothese aufgestellt, dass sie sich das große katabolische Potential der Mikroorganismen im Boden durch gezielte Aktivierungsmaßnahmen, z.B. die Abgabe von Schleimstoffen, für ihre eigene Verdauung zunutze machen (Barois, 1992; Lavelle et al., 1995; Trigo et al., 1999). Eine weitere potentiell symbiotische Assoziation von Regenwürmern (z.B. *Lumbricus terrestris*) mit Bakterien wurde erst kürzlich beschrieben: In den Nephridien sollen *Acidovorax*-ähnliche Bakterien am Proteinabbau beteiligt sein (Schramm et al., 2003).

1.4 Molekulare Diversitätsanalysen im Intestinaltrakt von Boden-Invertebraten

Kultivierungsunabhängige Diversitätsanalysen mit dem 16S rRNA-Gen als phylogenetischem Marker haben in den letzten Jahren auch in Intestinalsystemen eine große Anzahl bislang nicht kultivierter Mikroorganismen entdeckt, z.B. im Schweinedarm (Leser et al., 2002), im Pansen (Whitford et al., 1998), aber auch im menschlichen Darm (Suau et al., 1999). Der Schwerpunkt molekularer Diversitätsstudien an Boden-Invertebraten lag bislang bei Holz fressenden Termiten (z.B. (Hongoh et al., 2003; Ohkuma und Kudo, 1996; Shinzato et al., 1999), s. auch Arbeiten zitiert in (Brune und Friedrich, 2000; Ohkuma, 2003)). Erst kürzlich kamen umfassende Untersuchungen an Boden fressenden Termiten hinzu (Friedrich et al., 2001; Schmitt-Wagner et al., 2003a; Schmitt-Wagner et al., 2003b). Intestinaltrakte anderer Boden-Invertebraten wurden nur vereinzelt mit molekularen Methoden untersucht, z.B. bei Asseln (Kostanjsek et al., 2002) oder Regenwürmern (Schönholzer et al., 2002).

Für molekulare Diversitätsanalysen wird in der Regel der sog. „full-circle“ rRNA-Ansatz (Amann et al., 1995) favorisiert. Dieser umfasst die Klonierung und Sequenzierung mittels

PCR aus Umwelt-DNA-Extrakten amplifizierter 16S rRNA-Gene, die vergleichende phylogenetische Sequenzanalyse aller erhaltener Klone sowie die anschließende Formulierung von Sonden, die eine direkte Detektion einzelner Zellen in Umweltproben ermöglicht. Da ein Klonierungsansatz immer nur eine limitierte Anzahl von Klonen umfasst, werden bei Diversitätsstudien zunehmend auch molekulare Fingerprint-Techniken wie DGGE (Muyzer, 1999) oder T-RFLP (Kitts, 2001) eingesetzt, die die genetische Diversität eines Systems in ihrer Ganzheit abbilden können.

Molekulare Diversitätsanalysen bieten sich gerade für Untersuchungen an Invertebratendärmen an, weil es sich hierbei um sehr kleinräumig strukturierte, von steilen Gradienten (z.B. pH, O_2, H_2) geprägte Systeme handelt (Brune, 1998; Brune und Friedrich, 2000), die experimentell kaum zu simulieren sind. Kultivierungsansätze zeigen daher leicht ein verzerrtes Bild tatsächlich relevanter und numerisch abundanter mikrobieller Populationen (Liesack et al., 1997). Allerdings müssen auch die Ergebnisse molekularer Diversitätsstudien vorsichtig interpretiert werden, insbesondere wenn sie PCR-abhängige Teilschritte enthalten, bei denen Gene aus komplexen Umwelt-DNA-Gemischen amplifiziert werden ((Polz und Cavanaugh, 1998; Suzuki und Giovannoni, 1996), Übersicht bei (von Wintzingerode et al., 1997)).

1.5 Fragestellungen und Ziele der Arbeit

Im Rahmen dieser Arbeit wurden Struktur und räumliche Verteilung mikrobieller Gemeinschaften (v.a. *Bacteria* und *Archaea*) im Verdauungstrakt ausgewählter Boden-Invertebraten mit Methoden der molekularen mikrobiellen Ökologie untersucht, um die Funktion der Darmflora bei der Transformation organischer Substanz aus dem Boden, aber auch für die Ernährung der Tiere selber, besser zu verstehen. Im Mittelpunkt standen dabei Vergleiche der mikrobiellen Gemeinschaften von aufgenommenem Futter und Darm, von verschiedenen Darmabschnitten (z.B. Mittel- und Enddarm) und von verschiedenen Fraktionen eines Darmabschnittes (z.B. Enddarmlumen und -wand). Ein Vergleich der mikrobiellen Gemeinschaften von Darm und Futter erlaubt wichtige Schlüsse über die Spezifität der Darmflora, Kenntnisse über die räumliche Verteilung der Mikroorganismen innerhalb eines Darmsystems sind unerlässliche Grundvoraussetzung für ein tieferes Verständnis der Funktion einzelner Populationen.

Hauptuntersuchungsobjekte waren Boden bewohnende Scarabaeidenlarven („Engerlinge“), deren Darmflora molekularbiologisch bislang noch nicht untersucht wurde: humivore Larven des Kongo-Rosenkäfers (*Pachnoda ephippiata*) und phytophage Larven des Feldmaikäfers (*Melolontha melolontha*). Alle Untersuchungen an Scarabaeiden fanden in enger Zusammenarbeit mit der AG von Priv.-Doz. Dr. Andreas Brune (Universität Konstanz, jetzt MPI Marburg) statt, dessen Mitarbeiter einen großen Teil der physiko-chemischen Parameter in den Darmabschnitten bestimmten und damit eine wichtige Grundlage für die Interpretation der Zusammensetzung der Darmflora lieferten.

Die Untersuchungen an Scarabeidenlarven wurden ergänzt durch molekulare Untersuchungen am Regenwurm *Lumbricus terrestris*, dessen Darmsystem sich grundsätzlich von dem der Scarabaeidenlarven unterscheidet. In Zusammenarbeit mit der AG von Prof. Dr. Stefan Scheu (TU Darmstadt) wurde der Einfluss der Darmpassage auf die mikrobiellen Gemeinschaften im gefressenen Boden unter zwei verschiedenen Fütterungsbedingungen untersucht. Außerdem wurde nach Hinweisen für eine Darm-spezifische Flora im Verdauungstrakt von *L. terrestris* gesucht.

Weiterhin wurden im Rahmen dieser Arbeit molekulare Artefakte („pseudo-T-RFs“) entdeckt und beschrieben, die bei der T-RFLP-Analyse mikrobieller Gemeinschaften auftreten und zu einer Überschätzung der Diversität in untersuchten Proben führen können. Es wurde eine Methodik zur Identifizierung dieser Artefakte entwickelt und angewendet sowie Wege aufgezeigt, sie bei der Auswertung von T-RFLP-Profilen zu berücksichtigen. Außerdem wurde ein Protokoll entwickelt, mit dem sich bereits die Entstehung von pseudo-T-RFs nahezu verhindern lässt.

2 Material und Methoden

2.1 Chemikalien und Gase

Alle verwendeten Chemikalien wurden, soweit im Text nicht gesondert aufgeführt, im Reinheitsgrad „zur Analyse“ oder in vergleichbaren Reinheitsgraden von folgenden Firmen bezogen: Fluka (Buchs, Schweiz), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) und Sigma-Aldrich (Steinheim). Enzyme und molekularbiologische Reagenzien wurden von Amersham Pharmacia Biotech (Freiburg), Applied Biosystems (Weiterstadt), New England Biolabs (Frankfurt a.M.), Promega (Mannheim) und Roche Diagnostics (Mannheim) geliefert. Oligonukleotide wurden bei MWG-Biotech (Ebersberg) synthetisiert. Gase wurden von Messer-Griesheim (Darmstadt) bezogen.

2.2 Versuchstiere und ihre Präparation

2.2.1 Scarabaeidenlarven

Humivore Larven des Kongo-Rosenkäfers (*Pachnoda ephippiata* Gerstäcker 1867; *Coleoptera*, *Scarabaeidae*, *Cetoniinae*) wurden von der AG Brune (Universität Konstanz, jetzt MPI Marburg) bezogen. Larven und Käfer wurden in zwei 100 l-Terrarien bei ca. 27°C und einem 12 h-Tag/Nacht-Rhythmus aufgezogen (Abb. 2.1). Feuchtigkeit wurde täglich über einen Handvaporisator zugefügt.

Abbildung 2.1: Imago von *Pachnoda ephippiata* (Kongo-Rosenkäfer), Terrarien zur Aufzucht der Käfer und Larven in Konstanz und die drei Larvenstadien (L1 – L3) von *P. ephippiata*. Maßstab: ca. 1 cm. Foto der Larven: T. Lemke (Konstanz).

Unter diesen Bedingungen dauerte die Larvalentwicklung von *P. ephippiata* ca. 10 - 11 Wochen, wobei drei Larvenstadien (L1 - L3) durchlaufen werden (Abb. 2.1), die sich leicht anhand ihrer Kopfkapselgrößen unterscheiden lassen. Während die Käfer mit frischen Früchten und Salat gefüttert wurden, fraßen die Larven das im Terrarium befindliche Bodensubstrat, kommerzielle Blumenerden (Frux Blumenerde, Hagera, Reidersberg; Floragard Kompost, Floragard, Oldenburg) bestehend aus mäßig bis stark zersetztem Hochmoortorf, Grünschnittkompost, Tonmineralien (v.a. Montmorillionit) und anorganischen Salzen. Sorgfältig wurde darauf geachtet, dass die Larven keinen Zugang zum Käferfutter hatten.
Die untersuchten phytophagen Larven des Feldmaikäfers (*Melolontha melolontha* L.; *Coleoptera*, *Scarabaeidae*, *Melolonthinae*) waren Freilandfänge aus der Nähe von Obergrombach (bei Bruchsal), wo sie von Frühjahr 2002 bis Sommer 2003 als späte L2- bzw. L3-Stadien aus einem befallenen Weinberg bzw. Obstgarten ausgegraben wurden. In Marburg wurden die Larven über mehrere Monate einzeln in luftdurchlässig verschlossenen Plastikröhrchen bei ca. 15°C im Dunkeln gehalten (Abb. 2.2).

Abbildung 2.2: Larve von *Melolontha melolontha* (Feldmaikäfer; spätes L2-Stadium) und zur Hälterung der Larven in Marburg verwendete Plastikröhrchen.

Gefüttert wurden sie mit angefeuchteten Stückchen von Grassoden (Wurzeln von Wiesenkräutern und -gräsern in einer Bodenmatrix), die aus einer Wiese in der Nähe des Max-Planck-Instituts ausgestochen wurden. Zu Vergleichszwecken wurden in einer späteren Phase des Projektes *M. melolontha* Larven (L3) vier weiterer Standorte aus Deutschland und dem europäischen Ausland untersucht, die freundlicherweise von anderen Arbeitsgruppen zur Verfügung gestellt wurden (s. Kapitel 3.5). Dort wurden sie bei ca. 15°C in Torfsubstrat gehalten und mit Karottenstückchen gefüttert. Diese Larven wurden nicht für längere Zeit in Marburg gehalten, sondern gleich nach ihrer Ankunft seziert.

Alle Untersuchungen wurden mit L2-Stadien von *P. ephippiata* und L2/L3-Stadien von *M. melolontha* durchgeführt. Vor der Präparation wurden die Larven für ca. 20 min mit N_2/CO_2 (80/20, v/v) betäubt. Nach einer Vermessung (Länge, Gewicht etc.) wurden sie, die ventrale Seite nach oben und mit Nadeln fixiert, in einer Präparierwanne mit 4°C kalter, steriler Insekten-Ringerlösung (7,5g NaCl, 0,35g KCl, 0,21g $CaCl_2$ l^{-1}; (Brune et al., 1995)) mit sterilen Werkzeugen seziert. Nach der Dekapitation wurden die Larven an den Seitenlinien aufgeschnitten, das ventrale Integument entfernt, der Darm entnommen und von Fettzellen und Tracheen gereinigt. Mittel- und Enddarm (mit Rectum), die beiden Hauptabschnitte des Intestinaltraktes von Scarabaeidenlarven, konnten an ihrem Übergang leicht voneinander getrennt werden, ohne dass Darminhalt austrat (Abb. 2.3).

Abbildung 2.3: Verdauungstrakt einer Rosenkäferlarve (L3). Man erkennt die zwei Hauptabschnitte: Mitteldarm (mit 3 Kränzen von Blindsäcken) und Enddarm. Maßstab: ca. 1 cm. Aufnahme von T. Lemke (Konstanz).

Im Falle der Maikäferlarven wurden die einzelnen Darmabschnitte z.T. noch in Darmwand- und -lumenfraktion getrennt. Hierzu wurden die isolierten Därme geöffnet, Inhalt (Lumenfraktion) entnommen und die Darmwand anschließend mehrfach mit Ringerlösung gewaschen. Alle Fraktionen wurden gewogen und bis zur weiteren Verarbeitung bei -20°C eingefroren.

2.2.2 Regenwürmer

Die untersuchten Regenwürmer (adulte *Lumbricus terrestris* L.; *Oligochaeta*, *Lumbricidae*) stammten aus einem von der AG Scheu (TU Darmstadt) angesetzten Mikrokosmenexperiment. Es handelte sich um Freilandfänge aus dem Jägersburger Wald bei Darmstadt. Je drei Würmer wurden einzeln in mit Boden gefüllten Mikrokosmen mit und ohne Buchenstreu als

zusätzliche Futterquelle für drei Tage bei 20°C im Dunkeln inkubiert. Anschließend wurden die Würmer getötet, gewaschen und eingefroren. Aus den Mikrokosmen wurden Boden- und Losungsproben entnommen und ebenfalls eingefroren. Ziel des Experiments war, den Einfluss der Darmpassage auf die mikrobiellen Gemeinschaften des gefressenen Bodens unter zwei verschiedenen Fütterungsbedingungen mit molekularen Methoden zu untersuchen sowie nach Hinweisen für eine spezifische Flora im Regenwurmdarm zu suchen. Eine detaillierte Beschreibung des experimentellen Designs findet man in Kapitel 3.7.

Zur Präparation des Regenwurmdarms wurden die Tiere nach dem Auftauen mit Nadeln in einer Präparationswanne befestigt und mit sterilen Werkzeugen entlang des Dorsalgefäßes aufgeschnitten. Der Hautmuskelschlauch wurde unter Durchtrennung der einzelnen Septen vorsichtig nach beiden Seiten weggeklappt und der Darmkanal freigelegt. Der Darm hinter dem Muskelmagen (Darmwand mit Inhalt) wurde in jeweils drei gleich lange Abschnitte eingeteilt, von denen der mittlere (Mitteldarmabschnitte A und B nach (Horn et al., 2003)) für die molekularen Untersuchungen verwendet wurde. Alle Darmproben wurden bis zur weiteren Verarbeitung bei -20°C gelagert.

2.3 Chemisch-physikalische Analysen

2.3.1 Gaschromatographie (Methan)

Zur Bestimmung der Methanproduktionsraten von lebenden Maikäferlarven und isolierten Darmabschnitten wurde Methan an einem GC-8A Gaschromatographen (Shimadzu, Japan) mit FID-Detektor gemessen (*Trägergas*: Wasserstoff 5.0; *Säule*: 2 m Edelstahlsäule mit 1/8 Zoll Durchmesser und Propak QS 50/100 mesh als Trägermaterial, Temperatur: 40°C; *Detektor*: FID-Detektor mit H_2 und synthetischer Luft als Brenngase und N_2 als Quenchgas, Temperatur: 110°C) (Roy et al., 1997).

Larven bzw. frisch isolierte Mittel- und Enddärme wurden in 150 bzw. 36,5 ml Glasgefäßen unter Luft bzw. N_2-Atmosphäre für mehrere Stunden bei Raumtemperatur im Dunkeln inkubiert. Isolierte Darmabschnitte wurden mit etwas Ringerlösung inkubiert, um ein Austrocknen zu verhindern. Es wurde sorgfältig darauf geachtet, nicht zu viel Ringerlösung zuzugeben, um den Gasaustausch mit der Atmosphäre nicht zu behindern. In regelmäßigen Abständen wurden 100 µl Gasproben mit einer gasdichten Spritze (Dynatech Corp., Baton Rouge, USA) ent-

nommen und analysiert. Einpunkteichungen wurden mit Hilfe eines Gasgemisches aus jeweils 1000 ppmv CO, CO_2 und CH_4 in N_2 durchgeführt.

2.4 Molekularbiologische Methoden

2.4.1 DNA-Extraktion

DNA wurde aus Darm, Boden- und Losungsproben über eine direkte Lyse-Technik extrahiert, die auf der mechanischen Zerstörung von Zellen bei gleichzeitiger Anwesenheit denaturierender Reagenzien beruht (Henckel et al., 1999; More et al., 1994). Das zu extrahierende Material (ca. 0,1 - 0,5 g) wurde mit 0,7 g sterilen Zirkonium-Kugeln (0,1 mm Durchmesser; Biospec Products Inc., Bartlesville, USA), 700 µl Na-Phosphatpuffer (120 mM, pH 8) und 230 µl Natriumdodecylsulfat (SDS-)-Lösung (10% SDS; 0,5 M Na-Phosphatpuffer, pH 8; 0,1 M NaCl) in 2 ml Schraubdeckelgefäße überführt und in einer Zellmühle (FastPrep 120 Bead Beater, Qbiogene, Heidelberg) für 45 s bei maximaler Beschleunigung (6,5 m s^{-1}) aufgeschlossen. Nach Zentrifugation (21.000 *g*, 10 min, 4°C) wurden 600 µl des Überstandes in ein steriles 2 ml Gefäß überführt. Das Pellet wurde mit bis zu 600 µl frischem Na-Phosphatpuffer versetzt und ein zweites Mal extrahiert. Die vereinigten Überstande (ca. 1,2 ml) wurden mit 0,4 Volumenteilen 7,5 M NH_4-Acetat Lösung für 10 min auf Eis inkubiert, um Proteine und andere Verunreinigungen zu fällen, die anschließend abzentrifugiert wurden (21.000 *g*, 10 min, 4°C). Alternativ wurden Proteine und Verunreinigungen über eine Extraktion mit 500 µl Phenol/Chloroform/Isoamlyalkohol (25:24:1; v/v) abgetrennt. Zur optimalen Phasentrennung während der Zentrifugation (21.000 *g*, 5 min, 4°C) wurden 2 ml Phase Lock Gel Heavy Gefäße (Eppendorf, Hamburg) verwendet (Lüders und Friedrich, 2002). Aus den gereinigten, wässrigen Überständen (ca. 1 ml) wurde die DNA anschließend mit 0,7 Volumenteilen Isopropanol gefällt (Zentrifugation bei 21.000 *g* für 60 min bei Raumtemperatur). Die so erhaltenen Pellets wurden vorsichtig mit -20°C kaltem 70%igem Ethanol (ca. 250 µl) gewaschen, kurz abzentrifugiert und anschließend wenige Minuten unter Vakuum getrocknet. Danach wurden sie in 50 - 150 µl Puffer (10 mM Tris/HCl, pH 8,5) aufgenommen und der Erfolg der Extraktion durch Elektrophorese von 5 µl DNA-Extrakt in einem 1%igen Agarosegel und anschließende Ethidiumbromidfärbung kontrolliert.

Zusammen mit der DNA extrahierte Huminstoffe, die als PCR-Inhibitoren wirken (Wilson, 1997), wurden durch Adsorption an eine Polyvinylpolypyrrolidon (PVPP)-Matrix entfernt

(Berthelet et al., 1996; Henckel et al., 2000). 7,5 g PVPP wurden in 100 ml 3 M HCl durch Rühren über Nacht suspendiert. Anschließend wurde der pH-Wert der Suspension durch wiederholtes Sedimentieren (jeweils ca. 5 min) und Austauschen des Überstandes mit TE-Puffer (10 mM Tris, 1 mM EDTA, pH 8,0) auf pH 8 eingestellt. Zur Reinigung von DNA-Extrakten wurden, je nach Verunreinigungsgrad, 250 - 750 µl PVPP-Suspension in Mikro Bio-Spin Zentrifugen Kartuschen (Bio-Rad, München) gefüllt und in einem Ausschwingrotor (A-8-11 für Zentrifuge 5417 R, Eppendorf, Hamburg) für 1 min bei 380 *g* zentrifugiert. Die Benutzung des Ausschwingrotors bewirkte eine besonders einheitliche Schichtung des PVPPs in der Kartusche, wodurch die für eine ausreichende Reinigung von Extrakten benötigte PVPP-Menge (und damit der Verlust an DNA) deutlich reduziert werden konnte. Nach zweimaligem Äquilibrieren der PVPP-Säulen mit 100 µl Puffer (10 mM Tris/HCl, pH 8,5; Zentrifugation für 1 min bei 380 *g*) wurde der zu reinigende Extrakt vorsichtig auf die Säule aufgetragen und zentrifugiert (1 min, 380 *g*). Nach Kontrolle des DNA-Gehaltes über Gelelektrophorese wurden die klaren Eluate bis zur weiteren Verwendung bei -20°C gelagert.

2.4.2 PCR-Amplifikation von 16S rRNA-Genen

Zur Amplifikation bakterieller und archaeeller 16S rRNA-Gene wurden Domänen-spezifische Primer verwendet, deren Sequenzen in Tabelle 2.1 aufgeführt sind. Alle PCR-Ansätze wurden in einem Volumen von 50 µl durchgeführt. Das Standard-Reaktionsgemisch enthielt 1x PCR-Puffer (Applied Biosystems), 1,5 mM $MgCl_2$, 50 µM jedes Desoxyribonukleosidtriphosphats (Amersham), 0,5 µM beider Primer, 1,25 U AmpliTaq DNA-Polymerase (Applied Biosystems) und 1 µl Matrizen-DNA. Zur Vermeidung von Inhibitionseffekten, z.B. durch Huminsäuren, die durch die PVPP-Reinigung nicht vollständig entfernt werden konnten, wurde den PCR-Ansätzen ggf. bis zu 10 ng Rinderserumalbumin (Roche) zugesetzt (Kreader, 1996). Aus demselben Grund wurden die DNA-Rohextrakte zumeist verdünnt (1:10 - 1:100) als Matrizen eingesetzt. Alle Reaktionen wurden bei 4°C angesetzt, um unspezifische Primeranlagerungen zu verhindern. Die PCR-Reaktionen wurden in einem GeneAmp 9700 Thermocycler (Applied Biosystems) bzw. einem Mastercycler gradient (Eppendorf) mit folgendem Temperaturprofil standardmäßig durchgeführt: 3 min initiale Denaturierung bei 94°C, 32 - 35 Zyklen bestehend aus jeweils 30 - 45 s Denaturierung (94°C) und Primer-Anlagerung (52 - 55°C) sowie 60 - 90 s Extension (72°C), 5 - 7 min finale Extension bei 72°C. 5 µl Amplifikat wurde durch Standardgelelektrophorese mit anschließender Ethidiumbromidfärbung überprüft. PCR-

Amplifikate wurden mit dem MinElute PCR Purification Kit (Qiagen, Hilden) nach Anweisungen des Herstellers gereinigt.

Tabelle 2.1: Verwendete Primerkombinationen für die spezifische Amplifikation archaeeller und bakterieller 16S rRNA-Genfragmente aus den untersuchten Umweltproben.

Primer	Sequenz (5´→ 3´)	Produkt	Referenz
Ar109f	ACK GCT CAG TAA CAC GT	*Archaea* 16S rDNA	(Grosskopf et al., 1998)
Ar912r*	CTC CCC CGC CAA TTC CTT TA	(ca. 800 bp)	(Lüders u. Friedrich, 2000)
Ar109f	ACK GCT CAG TAA CAC GT	*Archaea* 16S rDNA	(Grosskopf et al., 1998)
Ar915r*	GTG CTC CCC CGC CAA TTC CT	(ca. 800 bp)	(Stahl und Amann, 1991)
27f*	AGA GTT TGA TCC TGG CTC AG	*Bacteria* 16S rDNA	(Edwards et al., 1989)
907r	CGG TCA ATT CTT TTR AGT TT	(ca. 900 bp)	(Muyzer et al., 1995)
27f	AGA GTT TGA TCC TGG CTC AG	*Bacteria* 16S rDNA	(Edwards et al., 1989)
1492r	TAC GGY TAC CTT GTT ACG ACT T	(ca. 1500 bp)	(Lane, 1991)

* Diese Primer wurden FAM-markiert in der T-RFLP-Analyse eingesetzt.

2.4.3 Erstellung von 16S rRNA-Gen Klonbibliotheken

Aus Umweltproben amplifizierte 16S rRNA-Genfragmente wurden über Klonierung vereinzelt, um ihre Sequenzierung und anschließende phylogenetische Einordnung zu ermöglichen. Gereinigte Amplifikate wurden dazu nach Herstellerangaben in den pGEM-T Vektor (Promega) ligiert und anschließend über hochkompetente *E. coli* JM109 Zellen (Promega) kloniert. Die transformierten Zellen wurden auf LB/Ampicillin/IPTG/X-Gal-Nährböden angezogen und über „Blau-Weiß-Kontrolle“ selektioniert. Aus den transformierten Zellen wurde DNA durch Aufkochen (10 min, 100°C) gewonnen. Die korrekte Länge der klonierten DNA-Inserts wurde anschließend über PCR mit Vektor-gerichteten Primern (Tab. 2.2) überprüft (Standardprotokoll mit 25 Zyklen und 55°C Annealing-Temperatur).

Tabelle 2.2: PCR-Primer für die Amplifikation klonierter DNA-Fragmente aus dem pGEM-T Vektor.

Primer	Sequenz (5´→ 3´)	Target	Referenz
M13f (-40)	TTT TTC CCA GTC ACG AC	pGEM-T	Promega
M13r (-48)	AGC GGA TAA CAA TTT CAC ACA GGA	Vektor	

2.4.4 Sequenzanalyse

Sequenzreaktionen wurden mit dem ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V2.0 mit AmpliTaq Polymerase FS (Applied Biosystems) durchgeführt. Das Reaktionsgemisch enthielt in einem Volumen von 10 µl ca. 75 ng amplifizierte Matrizen-DNA, 0,25 µM eines geeigneten Sequenzierprimers (Tab. 2.3, s. auch Tab. 2.1) und 3 µl BigDye Reaction Mix. Die Reaktion wurde nach 30 s Denaturierung bei 96°C mit 25 Zyklen (10s 96°C, 5s 50°C, 4 min 60°C) in einem GenAmp 9600 Thermocycler (Applied Biosystems) mit einer Heizrate von 1°C s^{-1} durchgeführt. Anschließend wurde das Reaktionsgemisch über AutoSeq G50-Gelfiltrationssäulen (Amersham) gereinigt, unter Vakuum getrocknet, in 2 µl Formamid Loading Dye (Amersham) re-eluiert, 3 min bei 90°C denaturiert und sofort auf Eis gekühlt.

Die Sequenzanalyse erfolgte auf einem ABI Prism 377 DNA Sequenzierer (Applied Biosystems). 1 µl DNA-Gemisch wurden auf ein 48 cm 4,25%iges PAGE-Plus Gel (nach Angaben des Herstellers) geladen und 18 h elektrophoretisch aufgetrennt (3000 V, 60 mA, 40 W).

Tabelle 2.3: Primer für Sequenzierreaktionen aus dem pGEM-T Vektor. Zur Sequenzierung von 16S rRNA-Gen -Inserts wurden z.T. auch die in Tabelle 2.1 aufgeführten Primer verwendet.

Primer	Sequenz (5´→ 3´)	Target	Referenz
M13 uni	CGA CGT TGT AAA ACG ACG GCC AGT	pGEM-T	Promega
M13 rev	CAG GAA ACA GCT ATG AC	Vektor	

Ein Teil der aus dem Maikäferdarm amplifizierten und klonierten 16S rRNA-Genfragmente wurde extern sequenziert (ADIS, Max-Planck-Institut für Züchtungsforschung, Köln). Hierzu wurden die transformierten *E. coli*-JM109 Zellen in einer 96er Mikrotiterplatte für mehrere Stunden bei Raumtemperatur in 2YT-F Medium (2YT-Medium + 10% (v/v) 10x Gefrierlösung; Tab. 2.4) inkubiert und anschließend tiefgefroren (-80°C) verschickt. Das im Medium enthaltene Glycerin verhinderte eine Zerstörung der transformierten Zellen beim Einfrieren.

Tabelle 2.4: Zusammensetzung von 2YT-Medium und 10x Gefrierlösung zur Herstellung von 2YT-F Medium.

Medium	Zusammensetzung
2YT	1,6% (w/w) Bacto-Trypton (Difco) 1,0% (w/w) Bacto-Hefeextrakt (Difco) 0,5% (w/w) NaCl
10x Gefriermedium	3 mM Magnesiumsulfat 15 mM Natriumcitrat 70 mM Ammoniumsulfat 55% (w/v) Glycerin 270 mM Di-Kaliumhydrogenphosphat* 130 mM Kaliumdihyrogenphosphat*

* getrennt vom Rest des Gefriermediums ansetzen, autoklavieren und anschließend unter sterilen Bedingungen mischen.

2.4.5 T-RFLP-Analyse

Standardprotokoll. Die T-RFLP-Analyse von 16S rRNA-Genen wurde mit FAM-(6-Carboxyfluorescein) markierten PCR-Produkten durchgeführt, die mit fluoreszenzmarkierten vorwärts-Primern (*Bacteria*) bzw. rückwärts-Primern (*Archaea*) nach einem Standard-PCR-Protokoll erzeugt wurden (s. Tab. 2.1). Gereinigte Amplifikate wurden einem Restriktionsverdau unterzogen, der standardmäßig folgende Zusammensetzung hatte: 50 ng (bei Klonen oder Reinkulturen) bzw. 75 ng (bei Umweltproben) PCR-Produkt, 2,5 U Restriktionsenzym und 1 µl 10x Reaktionspuffer in 10 µl Gesamtvolumen. Reaktionspuffer, Zugabe von BSA (1 µg) zum Verdau sowie die Temperatur, bei der anschließend für 3 h im Dunkeln verdaut wurde, richteten sich (entsprechend der Herstellerangaben) nach dem verwendeten Enzym. Bakterielle 16S rRNA-Genamplifikate wurden zumeist mit *Msp*I (Promega), archaeelle mit *Alu*I oder *Taq*I (Promega oder New England Biolabs) verdaut. Im Rahmen der Untersuchung von pseudo-T-RFs, PCR-abhängigen T-RFLP-Artefakten, die in dieser Arbeit erstmals beschrieben und in ihrer Entstehung näher untersucht wurden, wurden sowohl das zugrunde liegende PCR-Protokoll als auch viele Parameter des Restriktionsverdaus variiert. Auf diese Veränderungen wird detailliert in Kapitel 3.8 eingegangen.

Nach dem Verdau wurden 1 µl (bei Klonen oder Reinkulturen) bzw. 2,5 µl (bei Umweltproben) des Restriktionsansatzes und 0,8 µl GeneScan-1000 (ROX) Längenstandard (Applied Biosystems) vermischt und mit Formamid Loading Dye (Amersham) auf 5 µl aufgefüllt. Die-

se wurden dann 3 min bei 95°C denaturiert und sofort auf Eis gestellt. Die T-RFLP-Analyse wurde auf einem ABI Prism 373 DNA Sequenzierer (Applied Biosystems) im GeneScan Modus durchgeführt. 5 µl des Fragmentgemisches wurden auf ein 24 cm langes, 6%iges Polyacrylamid-Gel mit 8 M Harnstoff in 1x TBE Puffer (89 mM Tris-Borat, 2 mM EDTA) geladen und 6 h elektrophoretisch getrennt (2500 V, 40 mA, 27 W). Die Auswertung der Elektropherogramme erfolgte mit der zugehörigen GeneScan Analyse Software 2.1 (Applied Biosystems). Hierbei wurde die Länge der aufgetrennten Fragmente im Vergleich zum mit aufgetrennten internen Größenstandard sowie ihre Menge über die Stärke des Fluoreszenzsignals (ausgedrückt in Höhe oder Fläche eines jeden T-RF-Peaks) ermittelt. Hintergrundsignale (≤ 50 relative Fluoreszenzeinheiten) sowie T-RFs mit einer Länge ≤ 50 bp, die von Primer-Dimeren beeinflusst sein können, wurden grundsätzlich von der Analyse ausgeschlossen.
Vor einer statistischen Auswertung (s. Kapitel 2.6) wurden die T-RFLP-Profile i.d.R. auf gleiche Gesamt-Peakhöhe normalisiert. Dazu wurde ein iteratives Verfahren nach Dunbar (Dunbar et al., 2001) angewendet, bei dem wiederholt alle Peaks der miteinander zu vergleichenden Profile mit einem Faktor multipliziert werden, der von der niedrigsten aller Gesamt-Peakhöhen bestimmt wird. Sukzessiv wurden alle Peaks mit einer Höhe ≤ 50 Fluoreszenzeinheiten eliminiert, bis alle Profile die gleiche Gesamt-Peakhöhe aufwiesen.

Identifizierung und Vermeidung von pseudo-T-RFs. Pseudo-T-RFs sind PCR-abhängige Artefakte mit Auswirkungen auf die T-RFLP-Analyse mikrobieller Gemeinschaften, die in dieser Arbeit erstmals beschrieben wurden (s. Kapitel 3.8). Welche T-RFs in einem Profil von pseudo-T-RF-Bildung beeinflusst sind, ließ sich durch eine der T-RFLP-Analyse vorgeschaltete Behandlung des Amplikonpools mit DNA-Einzelstrang abbauender Mung bean-Nuklease feststellen. Hierzu wurden 1000 ng gereinigtes PCR-Produkt, 5 U Mung bean-Nuklease (New England Biolabs) und 10 µl 10x Puffer (New England Biolabs) in einem Volumen von 100 µl für 1 h bei 30°C inkubiert. Der Verdau wurde durch Zugabe von 100 µl Phenol/Chloroform/Isoamlyalkohol und Ausschütteln in 2 ml Phase Lock Gel Heavy Gefäßen (Eppendorf, Hamburg) gestoppt und die DNA aus der wässrigen Phase durch anschließende Fällung mit 2 Volumenteilen 96%igem Ethanol und 0,1 Volumenteilen 3 M Natriumacetat wiedergewonnen (Zentrifugation für 60 min bei 21.000 *g* und Raumtemperatur). Nach Trocknen unter Vakuum wurde die DNA in ca. 15 µl Puffer (10 mM Tris/HCl, pH 8,5) aufgenommen. Alternativ wurde der Nukleaseverdau durch Reinigung des Ansatzes mit dem MinElute

PCR Purification Kit (Qiagen) beendet. Mit den Nuklease-behandelten Amplikons wurde dann die T-RFLP-Analyse wie oben beschrieben durchgeführt. T-RF-Peaks, die in den mit Nuklease behandelten Profilen deutlich kleiner waren als in Profilen unbehandelter Amplikons bzw. sogar fehlten, wurden als die angesehen, die von pseudo-T-RF-Bildung beeinflusst sind.

Um herauszufinden, welche Amplikons für die pseudo-T-RF-Bildung in einem Profil verantwortlich sind, wurden alle Klone der zugrunde liegenden Klonbibliothek auf ihr in-vitro T-RF-Bildungsverhalten hin untersucht. Dazu wurde T-RFLP-Analyse mit klonaler DNA durchgeführt, die zuvor aus 1:10 verdünnten M13-Produkten mit 16 Zyklen amplifiziert worden war. Pseudo-T-RFs zeigten sich im Profil als zusätzliche Peaks (zum erwarteten T-RF), deren Länge einer zweiten oder höheren Schnittstelle in der Sequenz des jeweiligen Klons entsprach. Die Zuordnung von T-RFs in einem Umweltprofil zu Gruppen von Klonen in der zugrunde liegenden Klonbibliothek erfolgte anschließend nicht nur auf einer in-silico Analyse der klonalen Sequenzdaten, sondern auch auf Grundlage ihres in-vitro T-RF-Bildungsverhaltens und der Veränderung des Umweltprofils durch den Nukleaseverdau.

Da die Bildung von pseudo-T-RFs auf partiell einzelsträngigen DNA-Amplikons beruht, wurden Versuche mit Klenow-Fragment durchgeführt, um diese Amplikons vor dem Restriktionsverdau wieder aufzufüllen und so alle terminalen Schnittstellen dem Restriktionsenzym zugänglich zu machen. Hierzu wurden 1000 ng PCR-Produkt mit 5 U Klenow-Fragment Exonuklease Minus (Promega), 10 µl des zugehörigen 10x Puffers (Promega) und 50 µM eines jeden Desoxyribonukleosidtriphosphats (Promega) in einem Volumen von 100 µl für 1,5 h bei 25°C inkubiert. Die Behandlung wurde durch Reinigung mit dem MinElute PCR Purifcation Kit (Qiagen) gestoppt und die behandelten Amplikons einer T-RFLP-Analyse unterzogen. Anschließend wurden die Verhältnisse bestimmter T-RFs in Umweltprofilen aber auch in Profilen artifizieller Klongemische mit und ohne Klenow-Behandlung miteinander verglichen. Erwartet wurde, dass pseudo-T-RFs durch die Klenow-Behandlung eliminiert werden bzw. kleiner werden und gleichzeitig die zugehörigen primären T-RFs größer werden.

2.4.6 FISH

Zur Bestimmung absoluter Zellzahlen wurde FISH mit Cy3-markierten, gruppenspezifischen Oligonukleotidsonden (Tab. 2.5) in Anlehnung an ein Standardprotokoll durchgeführt (Pernthaler et al., 2001).

Tabelle 2.5: Verwendete rRNA-Oligonukleotidsonden; soweit nicht anders vermerkt, handelt es sich um 16S rRNA-Sonden, die mit 35% Formamid im Hybridisierungspuffer (s.u.) eingesetzt wurden.

Sonde[a]	Sequenz (5´→ 3´)	Zielgruppe[b]	Referenz
EUB338 I EUB338 II EUB338 III	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	*Bacteria*	(Daims et al., 1999)
ARCH915 ARC344	GTGCTCCCCCGCCAATTCCT TCGCGCCTGCTGCICCCCGT	*Archaea*	(Stahl und Amann, 1991) (Raskin et al., 1994)
SRB385	CGGCGTCGCTGCGTCAGG	Sulfat-Reduz.	(Amann et al., 1990)
DSV698-MK[c]	GTTCCTCCTGATCTCTACGG	*Desulfovibrio*	diese Arbeit
HGC69 A[d] Komp. HGC	TATAGTTACCACCGCCGT TATAGTTACGGCCGCCGT	*Actino-bacteria*	(Roller et al., 1994)
CF319 a CFB1082	TGGTCCGTGTCTCAGTAC TGGCACTTAAGCCGACAC	CFB-Phylum	(Manz et al., 1996) (Weller et al., 2000)
TM7905[e]	CCGTCAATTCCTTTATGTTTTA	TM7-Phylum	(Hugenholtz et al., 2001)
LGC A LGC B LGC C	TGGAAGATTCCCTACTGC CGGAAGATTCCCTACTGC CCGAAGATTCCCTACTGC	*Bacteria* mit niedrigem G+C-Gehalt	(Meier et al., 1999)

[a] systematische Sondennamen nach (Alm et al., 1996) findet man bei probeBase (http://www.microbial-ecology.net) (Loy et al., 2003).

[b] bezieht sich auf den gleichzeitigen Einsatz zusammen aufgeführter Sonden. Detailliertere Angaben zur tatsächlichen Spezifität der einzelnen Sonden sind über probeBase (s.o.) erhältlich.

[c] eine modifizierte DSV698 (Manz et al., 1998) Sonde (modifizierte Basen unterstrichen), um *Desulfovibrio*-Verwandte an der Enddarmwand von Maikäferlarven spezifisch zu detektieren.

[d] 23S rRNA-Sonde, wird zusammen mit einer Kompetitorsonde eingesetzt. 25% Formamid im Hybridisierungspuffer.

[e] 20% Formamid im Hybridisierungspuffer

Zu untersuchende Darmproben wurden mit einem Gewebehomogenisator (B. Braun Biotech International, Melsungen) in 10 ml 1x PBS-Lösung (130 mM NaCl, 10 mM Na_2HPO_4 / NaH_2PO_4, pH 7,4) homogenisiert. Das Homogenisat wurde anschließend 1:10 bis 1:100 verdünnt; Verdünnungen in dieser Stärke hatten sich für die nachfolgenden Zellzählungen bewährt. Zur Fixierung der Zellen wurden 1 ml verdünntes Homogenisat mit 3 ml 4%iger Paraformaldehyd-Lösung (w/v; in 1x PBS-Lösung) bzw. 1 ml 96%igem Ethanol über Nacht bei 4°C bzw. -20°C inkubiert. Anschließend wurden die fixierten Homogenisate mit einer Filtrationsanlage (Schleicher & Schüll, Dassel) auf weiße Polycarbonatfilter (25 mm Durchmesser,

0,2 µm Porengröße; Millipore, Eschborn) gebracht. PFA-fixierte Zellen wurden während der Filtration zweimal mit 1x PBS-Lösung gewaschen, um restliches PFA zu entfernen. Anschließend wurden die Filter luftgetrocknet und bei -20°C gelagert.

In-situ Hybridisierungen wurden mit dreieckigen Filterstückchen durchgeführt, die unmittelbar vor der Hybridisierung über eine aufsteigende Ethanolreihe (50%; 80%, 96%) für jeweils 3 min dehydriert wurden. Im Falle der Hybridisierung von Gram-positiven Zellen mit niedrigem G+C-Gehalt wurde vor der Entwässerung eine Lysozymbehandlung durchgeführt (Meier et al., 1999). Dabei wurden die Filter auf Eis für 4 min mit 50 µl einer Lysozymlösung (Sigma; 400 U μl^{-1}, in 100 mM Tris/HCl, 50 mM EDTA, pH 7,2) überstaut, die anschließend mit destilliertem Wasser abgewaschen wurde.

Nach Lufttrocknung wurde jedes Filterstück mit 20 µl eines Gemisches aus 2 µl 8 µM Sondenlösung (fluoreszenzmarkierte Oligonukleotidsonde in TE-Puffer) und 18 µl Hybridisierungspuffer (s.u.) überschichtet und für 90 min bei 46°C in einer feuchten Kammer im Dunkeln inkubiert. Wurden mehrere Sonden gleichzeitig eingesetzt, so wurde die Menge an Hybridisierungspuffer entsprechend reduziert. Als feuchte Kammern dienten verschließbare 50 ml Polyethylen-Röhrchen, in die ein mit ca. 2 ml Hybridisierungspuffer befeuchtetes Stück Zellstoff gelegt wurde.

Nach der Hybridisierung wurden die Filter für 20 min in 48°C warmen Waschpuffer (s.u.) gegeben und anschließend mit destilliertem Wasser gewaschen und luftgetrocknet. Die Zusammensetzung von Hybridisierungs- und Waschpuffer variierte je nach der (in der Literatur) für die einzelnen Sonden empfohlenen Formamidkonzentration, mit der die Stringenz der Hybridisierung eingestellt wird. In der Regel wurden alle Hybridisierungen mit 35%iger Formamidkonzentration durchgeführt. Die Zusammensetzung der entsprechenden Puffer zeigt Tabelle 2.6. Veränderte Formamidkonzentrationen im Hybridisierungspuffer wurden durch entsprechend geänderte Wassermengen ausgeglichen; die NaCl und EDTA-Konzentrationen im Waschpuffer wurden nach (Pernthaler et al., 2001) angepasst.

Zur DNA-Färbung wurden die luftgetrockneten, hybridisierten Filterstückchen mit 50 µl DAPI-Lösung (4´,6-Diamidino-2-phenlyindol; 10 µg ml^{-1} in 1x PBS-Lösung) für 5 min überstaut und anschließend mit destilliertem Wasser und 80%igem Ethanol gewaschen. Nach Lufttrocknung wurden sie zur Mikroskopie in mehrere Tropfen Citfluor Anti-Fading Reagenz (AF1 solution, Citifluor ltd., London, GB) eingebettet.

Tabelle 2.6: Zusammensetzung von Hybridisierungs- und Waschpuffer für FISH mit fluoreszenzmarkierten Oligonukleotidsonden bei 35% Formamidkonzentration.

Stamm-Lösung	Menge	Endkonzentration
Hybridisierungspuffer		
5 M NaCl	360 µl	900 mM
1 M Tris/HCl pH 7,4	40 µl	20 mM
Formamid	700 µl	35%
Aqua dest.	900 µl	
10% SDS (w/v)	2 µl	0,01%
Waschpuffer		
5 M NaCl	700 µl	70 mM
1 M Tris/HCl pH 7,4	1 ml	20 mM
0,5 M EDTA*	500µl	5 mM
Aqua dest.	ad 50 ml	
10% SDS (w/v)	50 µl	0,01%

* ab 20% Formamid im Hybridisierungspuffer hinzugeben

Die Auszählung der Zellen erfolgte an einem Epifluoreszenzmikroskop (Axiophot, Zeiss, Jena), ausgestattet mit Filtersätzen für DAPI und Cy3. Bei 1000-facher Vergrößerung wurden mindestens 1000 Zellen pro Filterstück mit Hilfe eines Zählgitters gezählt. Als positiv hybridisierte Zellen wurden nur solche Signale gewertet, die gleichzeitig ein Sonden- und DAPI-Signal zeigten. Die Ergebnisse der Zählungen wurden unter Berücksichtigung der ausgezählten Fläche und der Verdünnung des Homogenisates auf g FG homogenisierten Darm hochgerechnet.

Im Rahmen eines Einführungskurses in die konfokale Laser-Scanning-Mikroskopie (CLSM) unter Leitung von Dr. Thomas Neu (UFZ Leipzig-Halle, Magdeburg) wurden Aufnahmen vom mikrobiellen Biofilm an der Enddarmwand der Maikäferlarven gemacht. Hierzu wurde FISH mit kleinen Stückchen PFA-fixierter, von innen nach außen gestülpter Enddarmwand durchgeführt. Für die Hybridisierungen wurden Fluorescein-markierte EUBI und CY3-markierte SRB385w-Sonden bei 35% Formamid im Hybridisierungspuffer verwendet. SRB385w-Sonden (unpubliziert) sind modifizierte SRB385-Sonden mit einem Y-Wobble (C oder T) an Position 7 der Sondensequenz. Die hybridisierten Proben wurden zur Untersuchung unter dem CLSM (TCS-SP MP mit Leica Confocal Software 2.00 Build 0871; Leica,

Heidelberg) mit speziellem Silikonkleber (Dow Corning 3140 RTV Coating, Dow Corning Corp., Midland, USA) auf dem Boden kleiner Beobachtungskammern (20 mm breit, 2 mm tief; Coverwell Imaging Chambers, Electron Microscopy Sciences, Washington, USA) fixiert und dort in Citifluor-Lösung eingebettet, so dass die dreidimensionale Struktur des Biofilms erhalten blieb. Mikrofotografien (Kapitel 3.6) wurden mit einem 63 x 1.2 NA Wasser-Immersionsobjektiv aufgenommen, dreidimensionale Visualisierungen erfolgten mit der A-MIRA 2.2 Software (TGS, Merignac, Frankreich).

2.5 Bioinformatische Analysen

2.5.1 Sequenzverarbeitung

16S rRNA-Gen-Rohsequenzdaten wurden als Elektropherogramme mit dem Seqman II-Programm (DNASTAR, Madison, USA) einer Qualitätskontrolle unterzogen und editiert. Anhänge aus Vektorsequenzen wurden entfernt, Teilsequenzen und komplementäre Sequenzen wurden zu Gesamtsequenzen vereinigt. Die so erhaltenen Sequenzen wurden zu einer ersten phylogenetischen Einordnung mittels einer BLAST-Suche (Altschul et al., 1990) mit Einträgen in öffentlichen Datenbanken (z.B. http://www.ncbi.nlm.nih.gov/BLAST) verglichen. Die nächsten klonalen und kultivierten Verwandten wurden heruntergeladen und in die eigene 16S rRNA-Gendatenbank importiert.

2.5.2 Phylogenetische Analyse von 16S rRNA-Genen

Die phylogenetische Einordnung und Verrechnung von 16S rRNA-Gen-Sequenzdaten erfolgte mit Hilfe des Softwarepakets ARB (Version 2.5b; entwickelt von O. Strunk und W. Ludwig, TU München; erhältlich unter http//www.arb-home.de). Neu importierte Sequenzen wurden mit dem automatischen Fast Aligner (Vers. 1.03) mit anderen nahe verwandten Sequenzen in ein Alignment gebracht, d.h. homologe Nukleotidpositionen wurden untereinander in Spalten angeordnet. In der Regel wurden die Alignments noch nachträglich manuell korrigiert. Anschließend wurden die neuen Sequenzen über die „Quick Add Maximum Parsimony"-Funktion des ARB-Programms in den „Gesamtbaum" der Datenbank eingefügt. Zur Identifizierung von chimären Sequenzen, d.h. Mischprodukten aus Sequenzen phylogenetisch verschiedener Organismen, die als Artefakte während der PCR entstehen können, wurde ein sog. „fractional treeing" (Ludwig et al., 1997) durchgeführt: Ergab sich unter Berücksichtigung

der vorderen Hälfte einer Sequenz eine signifikant andere phylogenetische Einordnung als unter Berücksichtigung der zweiten Hälfte, so deutete dies auf eine chimäre Sequenz hin, die verworfen wurde.

Zur Berechnung phylogenetischer Bäume wurden allgemeine Basenfrequenzfilter für *Bacteria* und *Archaea* verwendet, die Bestandteil der ARB-Software sind. Der Einsatz solcher Filter beschränkt die phylogenetische Analyse auf solche Nukleotidpositionen, die, im Falle der hier verwendeten Filter, bei 50 - 100% aller *Bacteria* bzw *Archaea* konserviert sind. So werden hochvariable Regionen des 16S rRNA-Gens, deren phylogenetischer Informationsgehalt aufgrund hoher Mutationsraten fraglich ist, aus den Berechnungen ausgeschlossen.

Die im Rahmen dieser Arbeit berechneten phylogenetischen Bäume wurden auf Grundlage einer von der ARB-Software berechneten und evolutionär korrigierten Distanzmatrix, basierend auf einem paarweisen Vergleich aller zu verrechnender Sequenzen, mit Hilfe des Neighbor-Joining Algorithmus (Saitou und Nei, 1987) erstellt. In die Berechnungen gingen neben den eigenen Umweltsequenzen, die Sequenzen ihrer nächsten Verwandten aus öffentlichen Datenbanken sowie eine Vielzahl von Sequenzen bereits kultivierter *Bacteria* oder *Archaea* als Referenzsequenzen ein. Sequenzen, die deutlich kürzer waren als die Mehrzahl der Sequenzen, die für die phylogenetische Berechnung benutzt wurden (i.d.R. mind. 900 - 1500 bp), wurden erst nachträglich mit der „Quick Add Maximum Parsimony"-Funktion des ARB-Programms eingefügt, wodurch die generelle Topologie des erzeugten Baumes nicht mehr verändert wurde (Ludwig et al., 1998).

2.5.3 Entwicklung und Überprüfung von Oligonukleotidsonden

Gruppenspezifische Oligonukleotidsonden wurden mit Hilfe der in der ARB-Software implementierten Probe-design und Probe-match Programme entwickelt und getestet werden. Probe-design ermöglicht die Suche nach konservierten Gensequenzen, die phylogenetische Gruppen voneinander unterscheiden, mit Probe-match lässt sich abschätzen, welche Gruppen von Organismen mit bereits entwickelten Sonden detektiert und diskriminiert werden können.

2.5.4 Analyse der Sekundärstruktur von DNA-Fragmenten mit *mfold*

Um die Hypothese zu untersuchen, dass das in-vitro T-RF-Bildungsverhalten einzelner klonaler Amplikons von der Sekundärstruktur des in einem Teil der Amplikons vorhandenen, vermutlich mit sich selbst gefalteten DNA-Einzelstrangs abhängt, wurden potentielle Sekundär-

strukturen mit der online-Version von *mfold* (Zuker, 2003) untersucht (http://www.bioinfo.rpi.edu/applications/mfold). *Mfold* ist ein Programm, das unter Berücksichtigung externer Faktoren wie Temperatur und Salzkonzentration, Sekundärstrukturen einzelsträngiger RNA- und DNA-Moleküle aufgrund ihres Energiegehaltes vorhersagt, wobei die Strukturen mit dem geringsten Energiegehalt i.d.R. als die wahrscheinlichsten angesehen werden.

2.6 Statistische Methoden

2.6.1 Ökologische Indizes

Abschätzung des Artenreichtums. In Anlehnung an die Empfehlungen von Hughes et al. (Hughes et al., 2001) wurde der potentiell vorhandene Artenreichtum in den mit 16S rRNA-Gen Klonbibliotheken untersuchten Darmproben mit Hilfe von Chao1 (Chao, 1984) als Indikator [1] geschätzt. Dabei steht S_{obs} für die Anzahl tatsächlich gefundener Arten und n_1 bzw. n_2 für jeweils ein- bzw. zweimal gefundene Arten.

$$S_{Chao1} = S_{obs} + \frac{n_1^2}{2n_2} \qquad [1]$$

Ein Vergleich des geschätzten Artenreichtums mit der tatsächlich gefundenen Anzahl von Arten zeigt, inwieweit der klonale Ansatz mit seiner notwendigerweise beschränkten Anzahl von untersuchten Klonen die Diversität im untersuchten System erfasst hat. Als Arten wurden dazu Klone definiert, deren 16S rRNA-Gene mindestens zu 97% übereinstimmten (Stackebrandt und Göbel, 1994). Die Berechnungen der Schätzwerte von Chao1 erfolgten mit Hilfe von EstimateS (version 5.0.1; R. Colwell, University of Connecticut [http://viceroy.eeb.uconn.edu/estimates]).

Die Berechnung der 95%-Konfidenzintervalle der Schätzwerte erfolgte mit Hilfe von [2] aus (Chao, 1987), wobei S für Anzahl tatsächlich gefundener Arten, N für die geschätzte Anzahl von Arten (Chao1) und C für einen Faktor steht, der nach [3] berechnet wird. Die für [3] benötigte Standardabweichung σ wird von EstimateS zusammen mit den Chao1 Schätzwerten ausgegeben, die zugrunde liegende Formel findet man in (Hughes et al., 2001).

$$\left[S + (N - S)/C, S + (N - S)C\right] \qquad [2]$$

$$C = exp\left\{1{,}96\left[log\left(1 + \sigma^2/(N - S)^2\right)\right]^{0,5}\right\} \qquad [3]$$

95%-Konfidenzintervalle, die sich nicht überlappen, deuten auf signifikante Unterschiede zwischen zwei Schätzwerten hin.

Diversität und Ähnlichkeit von T-RFLP-Profilen. T-RFLP-Profile können untereinander mit Hilfe von ökologischen Indizes (Übersicht bei (Hill et al., 2003)) verglichen werden, wenn man die einzelnen T-RFs als Arten und ihre relative Höhe (bezogen auf die Gesamt-Peakhöhe eines Profils) als ein Maß für deren Häufigkeit interpretiert. Zum Vergleich der Diversität verschiedener Profile wurden der Shannon-Wiener Index H [4] und die (Shannon-) Evenness E [5] über die relativen Häufigkeiten der einzelnen T-RFs (p_i) und die Gesamtanzahl von T-RFs pro Profil (S) berechnet.

$$H = -\sum p_i \ln p_i \qquad [4]$$

$$E = \frac{H}{\ln S} \qquad [5]$$

Die paarweise Ähnlichkeit von T-RFLP-Profilen wurde in Anlehnung an Dollhopf et al. (Dollhopf et al., 2001) über Morisita-Indizes [6] verglichen, wobei n_i die Anzahl der Individuen von Art i, N die Gesamtanzahl aller Individuen und l den Dominanzindex nach Simpson [7] bezeichnet, wo s für die Gesamtanzahl der Arten in der untersuchten Gemeinschaft steht.

$$I_M = \frac{2\sum n_{1i} n_{2i}}{(l_1 + l_2) N_1 N_2} \qquad [6]$$

$$l = \frac{\sum_{i-1}^{s} (n_i(n_i - 1))}{N(N - 1)} \qquad [7]$$

Mit diesem Index werden zwei Gemeinschaften auf Grundlage des Auftretens von Arten sowie ihrer Häufigkeit verglichen. Morisitia-Indizes liegen zwischen 0 und 1, wobei 1 eine völlige (100%ige) Identität der beiden verglichenen Gemeinschaften andeutet.

Ergaben die Berechnungen der Indizes Unterschiede zwischen verschiedenen Profilen, so wurden diese mit nicht-parametrischen Tests (Mann-Whitney, Kruskal-Wallis) auf Signifikanz hin getestet. Auf gleiche Weise wurden auch Unterschiede zwischen anderen Parametern getestet, z.B. zwischen physiko-chemischen Parametern verschiedener Darmabschnitte. Alle Tests wurden mit Systat 10 (SPSS Inc., Chicago, USA) durchgeführt.

2.6.2 Mulivariate Statistik zur Analyse von T-RFLP-Profilen

Bei der Analyse komplexer T-RFLP-Datensätze bietet sich der Einsatz multivariater Statistikmethoden an, wenn es darum geht, die gleichzeitige Veränderung der relativen Häufigkeiten zahlreicher T-RFs in Abhängigkeit verschiedener Variablen übersichtlich darzustellen und auf ihre Signifikanz hin zu überprüfen (z.B. (Clement et al., 1998; Dollhopf et al., 2001)). Im Rahmen dieser Arbeit wurden, in enger Zusammenarbeit mit der AG von Stefan Scheu (TU Darmstadt), T-RFLP-Profile aus Futterboden, Darm und Losung von Regenwürmern mit einer Kombination aus multivariaten Methoden untersucht, die bislang noch nicht bei der Analyse von T-RFLP-Daten zum Einsatz kam.

Die Methode, eine Kombination aus Multidimensional scaling (MDS) und Discriminant function analysis (DFA), folgt einem von Puzachenko und Kuznetsov (Puzachenko und Kuznetsov, 1998) vorgeschlagenen Schema und wurde bereits zur Analyse von Pilzgemeinschaften eingesetzt (Tiunov und Scheu, 2000). Aus den relativen Häufigkeiten der (bakteriellen oder archaeellen) T-RF-Gruppen (OTUs) in den untersuchten Kompartimenten unter zwei Fütterungsbedingungen wurde zunächst eine Korrelationsmatrix aus nicht-parametrischen Gammakoeffizienten (analog zu Kendalls τ) berechnet, die anschließend einem MDS unterworfen wurde. MDS ist eine Ordinationstechnik, bei der Objekte in einem maximal 9-dimensionalen Raum so angeordnet werden, dass die Unterschiede zwischen ihnen möglichst gut widergespiegelt werden. Die Anzahl aussagekräftiger Dimensionen wurde durch einen Vergleich tatsächlicher Stresswerte mit der theoretischen, exponentiellen Stressfunktion ermittelt. Die Koordinaten der untersuchten Proben im n-dimensionalen Raum wurden mit DFA und „Kompartiment“ (Boden, Darm, Losung) als gruppierender Variable untersucht. Anschließend wurden Mahalanobis-Abstände (im Quadrat) zwischen den Gruppen-Centroiden sowie die Signifikanz der Gruppeneinteilung bestimmt. In der Regel ergaben sich zwei Diskriminanzachsen, so dass die DFA-Ergebnisse in zwei Dimensionen graphisch dargestellt werden konnten. Um abzuleiten, welche OTUs für die Trennung der Gruppen im Einzelnen verantwortlich sind,

wurden lineare Korrelationen zwischen den DFA Scores der einzelnen Proben und den relativen Häufigkeiten der OTUs berechnet. Alle Berechnungen wurden mit Statistica 6.0 (StatSoft, Hamburg) durchgeführt.

3 Ergebnisse

3.1 Physiko-chemische Bedingungen und mikrobielle Aktivitäten im stark alkalischen Darm humivorer Rosenkäferlarven

Thorsten Lemke, Ulrich Stingl, Markus Egert, Michael W. Friedrich und Andreas Brune

Anmerkung: Diese Arbeit war Bestandteil der Diplomarbeit von Thorsten Lemke (AG Brune, Universität Konstanz). Mein Beitrag bestand in der Erhebung absoluter Zellzahlen für die Mittel- und Enddarmabschnitte untersuchter Larven. Die Arbeit wird an dieser Stelle präsentiert, weil sie mit Daten zu Physikochemie und mikrobiellen Aktivitäten im Darm von *Pachnoda ephippiata* Larven wichtige Grundlagen für die Interpretation der kultivierungsunabhängigen Diversitätsstudie über die Darmflora dieser Tiere lieferte (Kapitel 3.2).

Zusammenfassung: Messungen mit Mikrosensoren zeigten, dass der Mitteldarm untersuchter *Pachnoda ephippiata* Larven z.T. extrem alkalisch (pH >10,0) ist. Beide Haupt-Darmabschnitte waren größtenteils anoxisch, angesichts des hohen pH-Wertes war das Redoxpotential im Mitteldarm sogar bei den dritten Larvenstadien noch relativ hoch. Nur im Enddarm traten bei allen drei Larvenstadien konsequent reduzierende Bedingungen auf (ca. –100 mV). Mittel- und insbesondere Enddarm waren dicht mit Mikroorganismen besiedelt, mikrobielle Fermentationsprodukte wurden in hohen Konzentrationen nachgewiesen. Eine Stimulation der Methanogenese im Enddarm durch exogene Elektronendonatoren wie H_2, Methanol und Formiat sowie das gleichzeitige Vorkommen von Formiat in Mitteldarm und Hämolymphe lassen vermuten, dass die Methanogenese im Enddarm durch einen Formiattransport aus dem Mitteldarm über die Hämolymphe angetrieben wird. Mit kultivierungsabhängigen Methoden konnten hohe Titer lactogener, acetogener und propionigener Bakterien nachgewiesen werden, die gut mit dem Spektrum an Gärprodukten in den jeweiligen Darmabschnitten sowie den Ergebnissen der kultivierungsunabhängigen Diversitätsstudie (Kapitel 3.2) übereinstimmen.

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Physicochemical Conditions and Microbial Activities in the Highly Alkaline Gut of the Humus-Feeding Larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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The soil macrofauna plays an important role in the carbon and nitrogen cycle of terrestrial ecosystems. In order to gain more insight into the role of the intestinal microbiota in transformation and mineralization of organic matter during gut passage, we characterized the physicochemical conditions, microbial activities, and community structure in the gut of our model organism, the humus-feeding larva of the cetoniid beetle *Pachnoda ephippiata*. Microsensor measurements revealed an extreme alkalinity in the midgut, with highest values (pH > 10) between the second and third crown of midgut ceca. Both midgut and hindgut were largely anoxic, but despite the high pH, the redox potential of the midgut content was surprisingly high even in the largest instar. However, reducing conditions prevailed in the hindgut paunch of all instars ($E_h \sim -100$ mV). Both gut compartments possessed a pronounced gut microbiota, with highest numbers in the hindgut, and microbial fermentation products were present in high concentrations. The stimulation of hindgut methanogenesis by exogenous electron donors, such as H_2, formate, and methanol, together with considerable concentrations of formate in midgut and hemolymph, suggests that midgut fermentations are coupled to methanogenesis in the hindgut by an intercompartmental transfer of reducing equivalents via the hemolymph. The results of a cultivation-based enumeration of the major metabolic groups in midgut and hindgut, which yielded high titers of lactogenic, propionigenic, and acetogenic bacteria, are in good agreement not only with the accumulation of microbial fermentation products in the respective compartments but also with the results of a cultivation-independent characterization of the bacterial communities reported in the companion paper (M. Egert, B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich, Appl. Environ. Microbiol. 69:6659–6668, 2003).

The soil macrofauna plays an important role in the carbon and nitrogen cycle of terrestrial ecosystems (18, 41, 62). The intestinal tracts of litter-feeding and humivorous soil macroinvertebrates are favorable habitats for microorganisms and typically harbor a dense and active gut microbiota. The major function commonly attributed to the microorganisms in the guts of such animals is the depolymerization and fermentative breakdown of the cellulosic or lignocellulosic component of their diet, which leads to degradation products that can be resorbed by the host. This is supported by the high concentrations of microbial fermentation products and by the presence of fermentative bacteria and protozoa, accompanied by obligately anaerobic homoacetogenic and methanogenic microorganisms, in the guts of such animals. However, the extent and importance of such processes and their specific function in the nutrition of the host are scarcely understood (for reviews, see references 8, 9, 11, 12, 14, 36, and 44).

In the case of soil-feeding termites, host factors such as the extreme alkalinity of the anterior hindgut and the influx of oxygen seem to play a key role in sequestering organic matter from the inorganic soil matrix (37). The decrease of molecular weight and increase in solubility resulting from alkaline extraction and chemical oxidation render the organic matter accessible for digestion in subsequent, less-alkaline compartments (34, 35). Alkaline gut regions are encountered also in many representatives of other insect orders and seem to be connected with the dietary preferences of the respective taxa (Coleoptera, Diptera, and Lepidoptera; see references 15 and 32). Comparing several beetle larvae feeding on a lignocellulosic diet, Grayson (30) already had pointed out an apparent correlation between the degree of humification of the diet and the alkalinity of the intestinal tract. The highest pH values among beetle larvae were encountered among the Scarabaeidae (50, 52, 58, 60), which comprise species from humivorous, detritivorous, and coprophagous feeding guilds. Although scarab beetle larvae are among those few arthropods that have received at least a minimum of attention from a microbiological perspective (3, 4, 21, 23) and although their importance for the transformation of soil organic matter is undisputed, little is known about the composition of the gut microbial community and its role in the digestive process.

Using the humivorous larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae) as a model organism, we investigated the physicochemical conditions and microbial activities in the gut in order to gain more insight into the role of the intestinal microbiota in transformation and mineralization of organic matter during gut passage. Since a representative analysis of the microbial community structure requires covering also those populations that resist cultivation (14), the project also included a cultivation-independent approach, involving molecular cloning and fingerprinting techniques. These

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results are reported and discussed in the companion paper (28).

MATERIALS AND METHODS

Insects. *P. ephippiata* Gerstaecker 1867 was bred in a 100-liter terrarium filled 20 cm deep with soil and illuminated with a 120-W light bulb on a 12-h day-night cycle; the average temperature was around 27°C (22 to 30°C). To ensure a constant high humidity, the walls and the soil surface were sprayed with soft tap water on a daily basis with a hand vaporizer. The terrarium contained freshly cut twigs of *Lonicera nitida* to provide resting places for the beetles. Imagines were fed with fresh fruit (apples, bananas, and peaches) and Chinese lettuce provided in a high-rimmed bowl, so that the food was not accessible to the larvae. The larvae therefore thrived almost exclusively on the substratum, consisting of commercial preparations of formulated organic potting soil, composed of moderately to highly decomposed sphagnum peat, grass compost, and a clay substrate with a high proportion of montmorillonite. Instars (Fig. 1A) were easily differentiated by the diameter of their head capsules. The average fresh weights (minimum-to-maximum range) of the first to third larval instars were 71 (26 to 140) mg (n = 24), 374 (152 to 780) mg (n = 18), and 2,541 (516 to 4,091) mg (n = 48), respectively.

Gut preparation. For dissection, larvae were anesthetized by exposure to a N_2-CO_2 (80/20, vol/vol) gas atmosphere for about 15 min, decapitated with scissors, and fixed with steel pins (ventral side up) in a preparation dish filled with insect Ringer's solution (16). The cuticle was cut along the sidelines, and the ventral integument was removed, the circular muscles and trachea being carefully severed from the skin. After a circular incision around the anus, the intestinal tract was carefully removed from the body; the residual fat body tissue and trachea were removed with a blunt instrument. First- and second-instar larvae were prepared using a dissecting microscope.

For the separate incubation of midgut and hindgut, and for the preparation of gut homogenates, isolated guts were separated at the muscular midgut-hindgut junction into a midgut section, including the short foregut, and a hindgut section, including paunch, colon, and rectum (Fig. 1B to D); no ligation was necessary to prevent leaking of the gut contents. Unless mentioned otherwise, all data reported in this paper are based on the fresh weight of the animal or the respective gut section; dry weight determinations indicated an average water content of approximately 87% for both midgut and hindgut of all instars. Gut volumes were estimated by measuring the outer diameter of the respective gut sections at different points along the axis, with a dissecting scope equipped with an ocular grid, and approximating the shapes to various geometric figures (a combination of truncated cones and cylinders).

The fecal pellets produced by the third instar (when freshly voided) had an average weight of 12 mg and a water content of 50% (calculated from fresh weight and dry weight of 100 fecal pellets). They were produced at a rate of approximately 1.5 pellets h^{-1}. By using the average dry weight of the gut of the third instar (109 mg), it was estimated that a larva produces almost two gut equivalents of feces per day.

CH_4 production rates. Larvae were placed into 10-ml (first and second instar) and 120-ml (third instar) glass vials, which were sealed (under air) with rubber stoppers, and were incubated for several hours at room temperature in the dark. If individual gut compartments were tested, the vials received a small volume of insect Ringer's solution (0.2 or 1 ml [16]) to avoid desiccation of the samples; care was taken not to cover the segments with liquid in order to facilitate gas exchange with the headspace. At regular intervals, gas samples were taken and analyzed for methane by gas chromatography, following the procedure described elsewhere in more detail (53). Stimulation of methane emission was tested by supplementing the headspace with H_2 (20 kPa) or by adding methanol (5 mM) or sodium formate (10 mM) to the Ringer's solution after the basal rate of methane production had been established.

Microsensor measurements. Clark-type oxygen microsensors with guard cathodes (48) were constructed and calibrated as described previously (16). Polarographic hydrogen microsensors had the same principal design (61), except that the working electrode was coated with platinum black (27). Testing and calibration procedures were performed as previously described (27). Both microsensor types had 90% response times of <5 s and tip diameters of 10 to 15 μm. The stirring sensitivity of both electrodes was <1% of the signal obtained upon calibration in air-saturated water or at 20 kPa of H_2, respectively.

Capillary microelectrodes with ion-selective membranes (LIX type) with a 200- to 300-μm-pore-size membrane of hydrogen ionophore I-cocktail A (Fluka) were constructed using the design described by Revsbech and Jørgensen (49). The microsensors were equipped with an external casing filled with 1 M KCl to minimize electrical noise (33) and had tip diameters of 10 to 30 μm and 90% response times of <10 s. The calibration was performed in commercial pH standard solutions (pH 6 to 12) and showed a log-linear response in this range (15). Electrically shielded platinum redox microelectrodes with tip diameters of 10 to 20 μm and response times of 10 to 20 s were constructed and calibrated with freshly prepared quinhydrone solutions as previously described (27). In both cases, the working electrode was connected to a high-impedance electrometer amplifier ($R_i > 10^{14}$ Ω) via a low-noise coaxial cable, and electrode potentials were measured against an Ag-AgCl electrode that was in contact with the

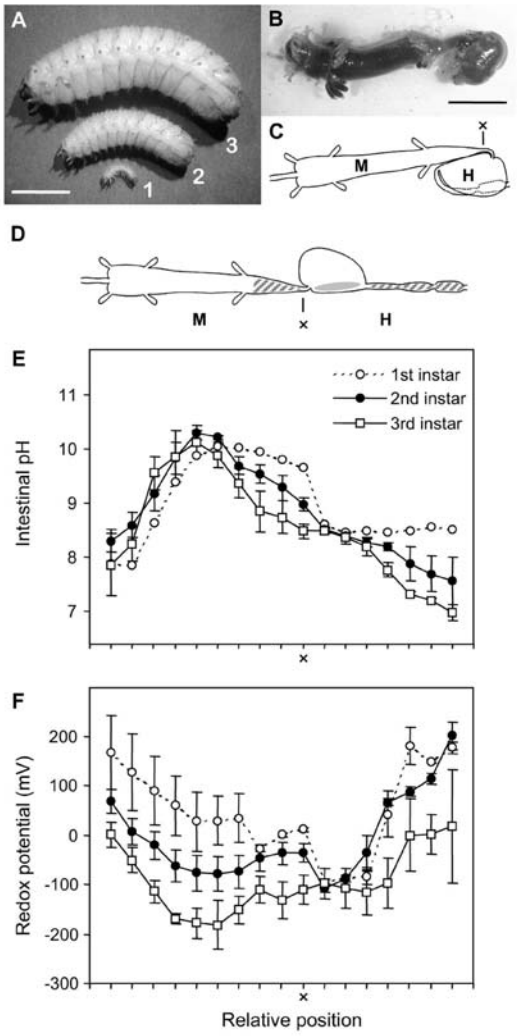


FIG. 1. Habitus of the larval instars (1 to 3) of *P. ephippiata* (A) and of the intestinal tract of the third instar (B and C), showing the three rings of gastric ceca and the point (×) where midgut (M) and hindgut (H) were separated. The gut habitus of the other instars is very similar (not shown). For microsensor measurements, guts were placed fully extended (D) into aerated insect Ringer's solution. Hatching indicates the gut regions of the first instar which were completely penetrated by oxygen, and shading indicates the only region of the hindgut where a slight accumulation of hydrogen was detectable (see text). Axial profiles of intestinal pH (E) and redox potential (F) were determined with microsensors. Bars (A and B), approximately 10 mm.

agarose-filled chamber via a KCl-filled agar bridge (1% agar in 1 M KCl). Reference and casing were grounded.

For the measurements, larvae were dissected, and the intact, fully extended gut (Fig. 1D) was immediately placed into a small polyvinyl chloride chamber (15 by 15 mm; 9 cm long) with glass front and back walls. A 5-mm layer of silicone cast into the bottom of the chamber allowed the gut to be fixed with minute steel pins. The chamber was irrigated with air-saturated insect Ringer's solution (16) by means of a peristaltic pump (5 ml min^{-1}). Measurements were carried out at ambient temperatures (20 to 25°C). Under these conditions, the dissected guts exhibited a moderate peristalsis that persisted for several hours, indicating that they were still physiologically active. Microsensors controlled by a manual micromanipulator were mounted vertically above the chamber; the progress of the tip was observed with a horizontally mounted stereomicroscope. A more detailed description of the setup has been published elsewhere (24).

Total cell counts. Cell numbers were obtained by a standard protocol (46) with the following modifications: gut sections were homogenized and appropriately diluted with phosphate-buffered saline solution and fixed with paraformaldehyde (3%, wt/vol) overnight at 4°C in the dark. Fixed homogenate (1 ml) was filtered onto white polycarbonate membrane filters, washed with phosphate-buffered saline, air dried, and subsequently stained with 4,6-diamino-2-phenylindole (DAPI). Cells were counted by epifluorescence microscopy with an ocular grid; approximately 1,000 to 2,000 cells were counted for each sample.

Metabolites in gut fluid. Individual gut compartments were blotted with tissue paper to remove residual Ringer's solution, transferred into plastic vials containing a defined volume of liquid (see below), and homogenized by using an ultrasonic probe (75 W, 20 s). After centrifugation (15 min at 14,000 × *g*), an aliquot of the supernatant was analyzed using the appropriate method (see below). All procedures for sample preparation were carried out at temperatures between 0 and 4°C.

For the determination of microbial fermentation products, gut sections were homogenized in 100 μl of HCl (400 mM) containing disodium malonate (5 mM) as internal standard, and supernatants were analyzed by high-pressure liquid chromatography (HPLC) (56). For the determination of total dissolved inorganic carbon (ΣCO_2), gut sections were homogenized in 100 μl of NaOH (5 mM), and supernatants were subjected to flow injection analysis (55). With gut sections of second and third instars, the volume of the homogenization fluid was increased to achieve at least a twofold dilution of the gut contents.

Metabolites in hemolymph. Hemolymph was collected from third instars directly after decapitation by using graded 100-μl capillaries; the yield was about 300 to 400 μl per larva. Samples were immediately transferred into the same volume of HCl (200 mM) containing phenylthiourea (2 mM) to prevent clogging (40), centrifuged (15 min at 14,000 × *g*), and analyzed by HPLC (56).

Serial dilution cultures. Midgut and hindgut sections were homogenized separately in sterile anoxic buffered salt solution with glass homogenizers. The homogenates were serially diluted (1:10) in substrate-free medium and inoculated (0.5 ml) into culture tubes containing medium (4.5 ml) amended with the respective substrates; the procedures have been described elsewhere in detail (56).

The medium for anaerobic cultivation was anoxic, bicarbonate-buffered mineral medium (AM-5 [7]), supplemented with yeast extract and Casamino Acids (Difco; 1 g $liter^{-1}$ each) and kept under a H_2-CO_2 gas mixture (80:20, vol/vol) at 50-kPa headspace pressure. The medium was reduced by adding a palladium catalyst (56) and adjusted to pH 7.0 with 1 M $NaHCO_3$. For cultivation at pH 10.0, $NaHCO_3$ was replaced by Na_2CO_3 and the concentrations of Ca^{2+}, Mg^{2+}, and PO_4^{3-} were reduced to 0.06, 0.05, and 0.14 g $liter^{-1}$, respectively, to avoid precipitation; the pH of the medium was adjusted by addition of 1 M Na_2CO_3.

The medium for aerobic cultivation (MM-5) was identical to medium AM-5 except that $NaHCO_3$ was replaced by sodium phosphate buffer (pH 7.0; final concentration, 20 mM; autoclaved separately), the concentrations of $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were decreased to 0.1 and 0.015 g $liter^{-1}$, respectively, and trace element solution SL 10 was replaced by trace element solution SL 11 (29). Medium for aerobic cultivation also received less yeast extract and Casamino Acids (0.5 g $liter^{-1}$ each).

Substrates were added from sterile stock solutions before the medium was dispensed into the dilution tubes. Substrate concentrations for aerobic cultures were 2.5 mM (D-glucose) and 5 mM (L-lactate and aromatic compounds). For anaerobic cultures, the amount of substrate was doubled. All cultures were incubated at 30°C in the dark; the tubes were slanted and gently agitated on a rotary shaker (100 rpm).

Metabolic product profiles. Growth was routinely ascertained by checking turbidity and testing substrate utilization and product formation by HPLC (56). Inoculated tubes were scored as positive if substrate degradation was confirmed by the results of HPLC analysis of the culture supernatant after 2 weeks (oxic) or 4 weeks (anoxic) of incubation. The presence of sulfate-reducing bacteria and methanogenic archaea was inferred from the production of sulfide (25) or methane (47). The presence of homoacetogens was indicated when acetate production was well in excess over the maximum amount theoretically formed by substrate oxidation. Utilization of aromatic compounds was analyzed by HPLC (13). To ensure that all major products were accounted for, electron balances were routinely determined for each tube (56).

Statistics. Unless mentioned otherwise, each data set represents the mean (±standard error) of the results obtained with at least four different animals. Significance was evaluated by one-way analysis of variance ($P < 0.05$).

RESULTS

Total cell counts. The intestinal tract of *P. ephippiata* larvae consists of two major compartments—a long, cylindrical midgut decorated with three rings of midgut ceca, and a bulbous hindgut (Fig. 1A to D). Both gut compartments are colonized by prokaryotic microorganisms, with cell densities of (8.9 ± 3.5) × 10^9 cells per g (fresh weight) in the midgut and (4.0 ± 1.4) × 10^{10} cells per g (fresh weight) in the hindgut (DAPI counts; second instar). Cell densities obtained for the third instar were in the same range; none of the values differed significantly due to the large individual variance. In all larvae, however, the hindgut values were significantly higher (three- to sixfold) than those for the midgut. Phase-contrast microscopy revealed the presence of cocci, rods, and filamentous morphotypes. Numerous ciliate protozoa were present in the hindgut but not in the midgut. Also nematodes were regularly encountered in the hindgut; in third instars, they reached a length of almost 10 mm, and up to 20 to 30 individuals per larva were observed.

Axial profiles of intestinal pH and redox potential. Microsensor measurements performed with intact intestinal tracts incubated in aerated Ringer's solution revealed that the guts of all instars were characterized by large dynamics of pH and redox potential (Fig. 1E and F). The intestinal pH increased sharply in the anterior midgut and reached maxima between pH 10.1 and 10.7 beyond the second crown of midgut ceca (Fig. 1E). The maximal values did not differ significantly among the instars (pH 10.2 ± 0.1, $n = 13$). Beyond the third crown of ceca, the pH declined again, and it remained quite constant at slightly alkaline values over the bulk of the hindgut (pH 8.4 ± 0.1, $n = 13$). Only in larvae of the second and third instars did the gut content return to neutral towards the rectum.

In contrast to the intestinal pH, the redox potential (E_h) of the midgut contents shifted considerably during larval development. The midgut of first-instar larvae was characterized by oxidizing conditions, whereas the E_h shifted to reducing conditions in larvae of the second and third instars (Fig. 1F). The contents of the anterior hindgut paunch showed reducing conditions in all instars ($E_h = -100 \pm 11$ mV, $n = 11$). In the posterior hindgut, the E_h shifted again to more positive values, especially in the smaller guts of the earlier instars.

Oxygen concentration profiles revealed steep gradients around the midgut and hindgut compartments; in no case did O_2 penetrate deeper than 100 μm into the lumen of the respective segments. The complete consumption of O_2 in the gut periphery led to anoxic conditions at the gut center of all instars (details not shown). Only in the small guts of the first instar did oxygen penetrate the thin tubular gut regions posterior to the third crown of ceca and posterior to the paunch (Fig. 1D). Hydrogen concentration profiles showed that the

TABLE 1. Methane production by the three instars of *P. ephippiata* larvae and by isolated midguts and hindguts (third instar) incubated separately in Ringer's solution under air

Larva or gut section	CH_4 production rate (μmol g^{-1} h^{-1})[a]	*n*
Larvae		
First instar	0.13 ± 0.04	4
Second instar	0.12 ± 0.03	4
Third instar	0.36 ± 0.11	6
Isolated gut sections		
Midgut	ND[b]	14
Hindgut	0.030 ± 0.007	14

[a] All values are based on the fresh weight of the whole animal and are means ± standard errors for *n* different larvae.
[b] ND, not detectable (<0.1 nmol g^{-1} h^{-1}).

accumulation of H_2 (approximately 60 to 70 Pa) was restricted to only a small region in the dorsal part of the hindgut paunch (Fig. 1D; details not shown), which is in direct contact with the posterior midgut in situ (Fig. 1C).

Gas exchange rates of larvae. Oxygen consumption rates of all instars were in the same range (~10 μmol g^{-1} h^{-1}), with a large individual variance. This might have been caused by differences in the activities of the individual larvae or by different developmental phases within an instar and was not further investigated. Also methane emission of individual larvae varied strongly (Table 1), with rates ranging between 0.05 and 0.45 μmol g^{-1} h^{-1}. In spite of all individual variance, however, the specific rates of methane formation were usually higher in the third instar.

Separate incubation of isolated midguts and hindguts of third instars revealed that CH_4 was produced exclusively by the hindgut (0.20 μmol g of hindgut^{-1} h^{-1}), which corresponds to less than 10% of the rate observed with living animals (Table 1). However, when isolated hindguts were incubated in the presence of potential methanogenic substrates, CH_4 formation was strongly stimulated (up to fivefold) over the endogenous rates. Interestingly, the stimulating effect of H_2 added to the headspace gas was much less pronounced than that caused by addition of methanol or formate to the incubation buffer (Table 2).

Microbial fermentation products. The intestinal tracts of all instars contained short-chain fatty acids and other metabolites typical of microbial fermentations. The total concentration of fermentation products in midgut and hindgut was significantly lower in the first instar than in the second and third instars (Table 3). The latter did not differ significantly from each other or from the total concentration of fermentation products in the hemolymph of the third instar (5.7 ± 1.2 mM [*n* = 5]). In all instars, midgut and hindgut contained high concentrations of inorganic carbon (ΣCO_2). While concentrations did not differ significantly among the instars, the concentrations in the midgut were significantly higher than those in the hindgut (Table 3).

TABLE 2. Stimulation of methane emission by isolated hindguts of *P. ephippiata* (third instar) incubated in Ringer's solution by the addition of external electron donors

Substrate added	CH_4 formation rate (μmol g^{-1} h^{-1})[a]		Stimulation (fold)[a]	*n*
	Basal	After addition		
H_2[b]	0.13 ± 0.03	0.21 ± 0.01	1.8 ± 0.4	3
Methanol[c]	0.15 ± 0.08	0.50 ± 0.20	4.3 ± 1.2	5
Formate[d]	0.28 ± 0.19	1.04 ± 0.50	4.9 ± 1.2	4

[a] All values are based on the fresh weight of the hindgut and are means ± standard errors for *n* different larvae.
[b] Headspace partial pressure of 20 kPa.
[c] Final concentration of 5 mM.
[d] Final concentration of 10 mM.

However, the spectrum of fermentation products differed considerably between midgut and hindgut, and also among the different instars (Fig. 2), which indicated changes in the composition of the intestinal microbiota during larval development. High concentrations of lactate and acetate were present in the midgut of all larvae, with acetate predominating in the first instar and lactate in the later instars. Also formate accumulated in the midgut of all larvae, with highest concentrations in the third instar. By contrast, acetate was the most abundant fermentation product present in the hindguts of all larvae and was accompanied by relatively high concentrations of propionate in all instars except the first. Interestingly, the composition of the hemolymph (determined only for the third instar) reflected the spectrum of microbial fermentation products in the midgut rather than that in the hindgut (Fig. 2).

Metabolic product profiles in serial dilutions. In order to identify the metabolic potential of the gut microbiota and the relative abundance of major metabolic groups, enrichment cultures on different substrates were inoculated with 10-fold serial dilutions of midgut and hindgut homogenates of a *P. ephippiata* larva (third instar) amended with various substrates. Growth was recorded, and metabolic product profiles were determined for all dilutions.

Under oxic conditions, all substrates were completely oxidized to CO_2, whereas microbial fermentation products accumulated under anoxic conditions. Figure 3 illustrates the results obtained for a dilution series with glucose as substrate. A comparison of the product profiles obtained with midgut and hindgut homogenates incubated at pH 7 indicates that the microbiota of the two gut sections differed strongly. For the midgut, the formation of propionate and butyrate was observed only in the lower dilutions, whereas the products of the higher dilutions consisted almost exclusively of lactate, acetate, ethanol, and formate. For the hindgut, however, butyrate formation was observed even in the highest positive dilution, and also propionate was a major product in all except the highest positive dilution. Moreover, the number of positive tubes was usually one tube higher than in the case of the midgut.

When midgut homogenates were serially diluted in alkaline medium (pH 10), the number of the highest positive dilution was always identical to that obtained with glucose at pH 7. While the higher dilutions had product profiles similar to those of the corresponding series in neutral medium (Fig. 3), no butyrate was formed and propionate formation was reduced in the lower dilutions; also growth was slower than under neutral conditions. Similar results were also obtained with Casamino Acids (details not shown). The same dilution depth was obtained under oxic conditions.

Table 4 summarizes the results obtained with dilution series on other substrates; the results allows an estimation of the

TABLE 3. Gut volume and total concentrations of fermentation products (converted to glucose equivalents) and dissolved inorganic carbon (ΣCO_2) in midgut and hindgut of the three instars of *P. ephippiata* larvae[a]

Larval instar	Vol (μl)			Fermentation products[b] (mM)			ΣCO_2[c] (mM)		
	Midgut	Hindgut	*n*	Midgut	Hindgut	*n*	Midgut	Hindgut	*n*
1	18 ± 1	14 ± 1	14	2.6 ± 0.6	1.4 ± 0.2	5	41 ± 11	27 ± 7	5
2	71 ± 9	74 ± 7	17	7.0 ± 2.7	6.2 ± 1.9	6	32 ± 8	24 ± 4	5
3	543 ± 43	398 ± 26	23	6.8 ± 1.3	7.5 ± 3.2	11	46 ± 8	23 ± 2	5

[a] Values are means ± standard errors for *n* different larvae.
[b] Based on the number of reducing equivalents released upon formal oxidation to CO_2.
[c] Total dissolved inorganic carbon (CO_2 plus HCO_3^- plus CO_3^{2-}).

abundance of the major metabolic groups in midgut and hindgut. The highest positive dilutions were consistently obtained for the hindgut compartment, both with glucose and with Casamino Acids as substrate, regardless of whether oxic or anoxic media were used for the enumeration. The apparent number of cellulose-degrading bacteria was inferred from the complete dissolution of filter paper disks incubated in the basal medium. It was 1 to 2 orders of magnitude lower than that obtained for bacteria degrading glucose or Casamino Acids, both in midgut and in hindgut. Cellulose degradation was observed only in oxic dilution series, and cellulose was not degraded under alkaline conditions.

Anaerobic bacteria were present both in midgut and in hindgut, although the apparent numbers were consistently higher in the hindgut (Table 4), where both lactogenic and butyrogenic physiotypes were present in the highest dilutions. Lactate-fermenting bacteria formed propionate and acetate as major products, and judging by the occurrence of propionate formation in the dilutions series on glucose, it appeared as if the same populations are also responsible for propionate formation in these cultures. Also methanogenic archaea seem to be present in similar abundance, whereas the apparent numbers of CO_2-reducing homoacetogenic bacteria and of lactate-oxidizing sulfate-reducing bacteria are considerably lower. Methane production was much lower in the alkaline series, whereas the apparent number of homoacetogens was unaffected by the pH of the medium. Aerobic bacteria mineralizing vanillate and cinnamate were present in considerable numbers, but aromatic compounds were not degraded under anoxic conditions; only demethylation of vanillate and side chain reduction of cinnamate occurred in lower dilutions.

DISCUSSION

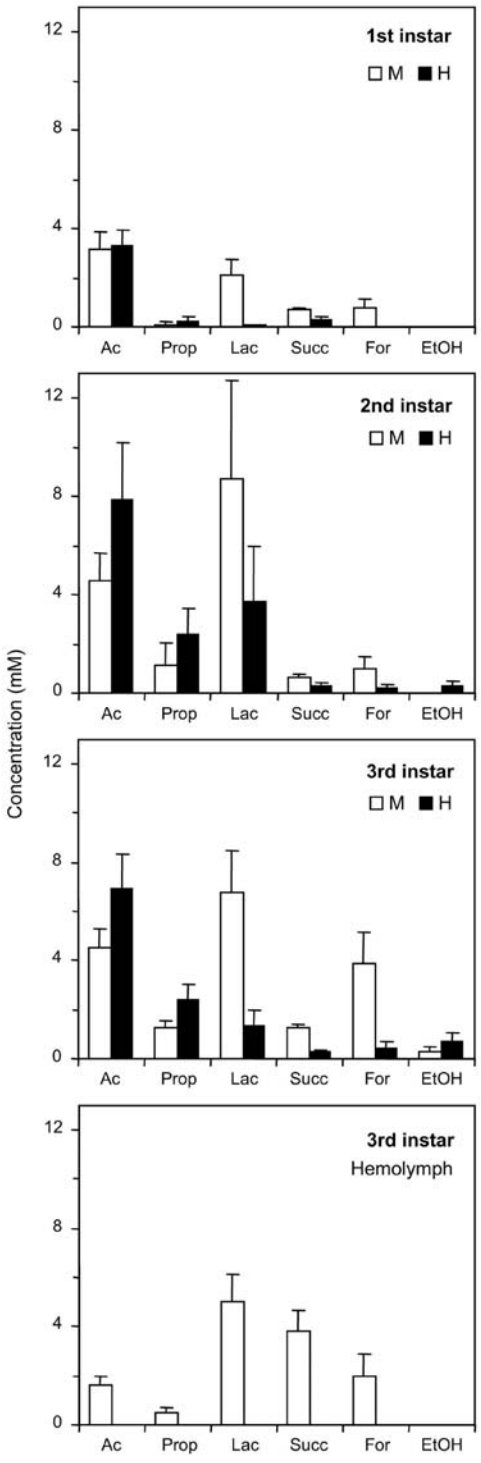
The importance of the gut microbiota in fiber digestion by scarabaeid beetle larvae was first recognized by Werner (59), who demonstrated cellulose degradation, proteolytic activities, and microbial fermentation products in the gut of the larva of *Potosia cuprea*. Later investigations revealed that the presence of a strongly alkaline midgut equipped with hydrolytic enzyme activities and a circumneutral hindgut characterized by microbial fermentation processes is typical for the larvae of the Scarabaeideae (3, 4, 30, 50, 60).

Physicochemical gut conditions. The first reports on the extremely alkaline pH in the midgut of scarabaeid beetle larvae date back almost 80 years (52, 58, 60). Grayson (30) compared the gut pHs of different coleopteran larva and found highest pH values in the midgut contents of *Osmoderma scabra* (pH 10.2) and *Cotnis nitida* (pH 10.1), which feed on decaying logs or humic soil; he carefully concluded that "those species feeding on decaying organic matter have a medium to strong alkaline reaction in the midgut."

The fine-scale intestinal pH profiles presented in this study document a pronounced dynamic of physicochemical conditions along the gut of *P. ephippiata*. The results are in good agreement with those reported for third-instar larvae of *Costelytra zealandica* (5), where the pH increased strongly in the prececal portion, remained rather constant (pH 10.8 to 10.9) in the anterior and middle portion, and dropped at the posterior end of the midgut, whereas a constant pH of 8.2 was present in the large hindgut. A similar situation seems to be present also in *Oryctes nasicornis*, where the midgut has a pH of 11.7 and the pH in the hindgut is 8.5 (3). The mechanism responsible for the generation of the alkaline pH remains to be investigated; it may be similar to that described for certain lepidopteran larvae (26, 32).

Although the midgut of *P. ephippiata* is mostly anoxic, most likely owing to the combination of microbial respiration and chemical oxygen consumption of the alkaline gut contents, as in soil-feeding termites (37), and highly alkaline, it has a relatively positive redox potential, whereas the neutral hindgut is more reduced. The E_h values are similar to those reported for the larva of *Oryctes nasicornis* (+30 mV in the alkaline midgut and −80 to −100 mV in the neutral hindgut [3]). Slightly lower redox potentials have been reported for midgut and hindgut of *Pachnoda marginata* (−100 to −200 mV [19]). The situation in humivorous beetle larvae is similar to that in soil-feeding termites (*Cubitermes* spp.), where the intestinal redox potential appears to be controlled not only by the presence of oxygen or hydrogen and the prevailing pH in the respective gut compartments but also by other redox-active components, such as humic substances and iron minerals, which are potential mediators and/or electron acceptors for the mineralization of organic matter (38). Interestingly, the conditions in the gut of the two smaller instars of *P. ephippiata* were slightly more oxidizing than those in the large gut of the third instar. This effect is probably related to the larger surface-to-volume ratio in the small instars, which should increase the rate of oxygen influx via the gut epithelium (11). Oxygen reduction in the gut periphery has been shown to affect the metabolic processes in the gut of *Reticulitermes flavipes* (55) and would also explain the differences in the fermentation product patterns between the instars of *P. ephippiata* (Fig. 2).

Hydrolysis of polymers. Many scarabaeid beetle larvae can feed exclusively on humus, e.g., on a peat soil devoid of living



plant roots. An analysis of the gut contents has revealed that larvae of *Adoryphorus couloni* feed preferentially on the organic soil constituents, which are sequestered at two to four times their concentration in the bulk soil (43). In *P. ephippiata*, the weight of the gut represents almost half the larval biomass (51, 44, and 38% in the first, second, and third instars, respectively), and based on dry weight, captive larvae produce almost two gut equivalents of feces per day.

The high pH in the midgut should facilitate the desorption of humic substances from the mineral matrix, thus rendering organic components accessible to enzymatic digestion (37).The black-brown contents of the alkaline midguts of *Cetonia aurata* and *Potosia cuprea* (pH 11 to 11.5) contain alkaline protease and amylase activities, whereas cellulase activities seem to be restricted to the hindgut (pH 7.0) (52, 58). In their investigations of *Oryctes nasicornis*, Bayon and Mathelin (4) have shown high rates of cellulose hydrolysis in midgut and hindgut, and also *P. marginata* larvae possess high activities of hydrolytic enzymes (xylanase and carboxymethyl cellulase) in midgut and hindgut (20). High protease activity has been reported for the midgut of third instars of *Costelytra zealandica* (5), and also the midgut of *Melolontha melolontha* larvae contains a complex mixture of proteinases, recently described in detail (57). Numerous alkali-stable proteases are also found in *P. ephippiata* (H. Zhang and A. Brune, unpublished results), and high ammonium concentrations in the hindgut of *P. ephippiata* (T. Lemke, X. Li, and A. Brune, unpublished results) indicate that the products of enzymatic hydrolysis are eventually subject to degradation by the animal and/or its gut microbiota.

Microbial fermentations. The exact contribution of the gut microbiota to the hydrolysis of different dietary components remains to be clarified since both the host and its microbial symbionts are potential sources of digestive enzymes. Nevertheless, the participation of gut microorganisms in the fermentative breakdown of the products of enzymatic hydrolysis is clearly evidenced by the high concentrations of microbial fermentation products in the midgut and hindgut fluid. The relative abundance of fermentation products in midgut and hindgut of *P. ephippiata* differs considerably and also changes during larval development. A dominance of lactate and acetate among the fermentation products in midgut and hindgut has been reported for *P. marginata* (20, 21), whereas acetate and propionate are the major metabolites in *Oryctes nasicornis* (3, 4).

As in other insects possessing a digestive microbiota, the microbial fermentation products will eventually be oxidized by the animal (10). This is in agreement with the presence of short-chain fatty acids in the hemolymph, which has been reported also for the larva of *Oryctes nasicornis* and *Popillia japonica* (3, 54). Although the pattern of metabolites in the hemolymph resembles that of the midgut more closely than

FIG. 2. Microbial fermentation products in midgut (M) and hindgut (H) of the three larval instars and in the hemolymph (third instar) of *P. ephippiata*. Concentrations were calculated using the geometrically estimated volumes. Bars represent standard errors for five separate guts ($n = 11$ for the third instar). Abbreviations: Ac, acetate; Prop, propionate; Lac, lactate; Succ, succinate; For, formate; EtOH, ethanol.

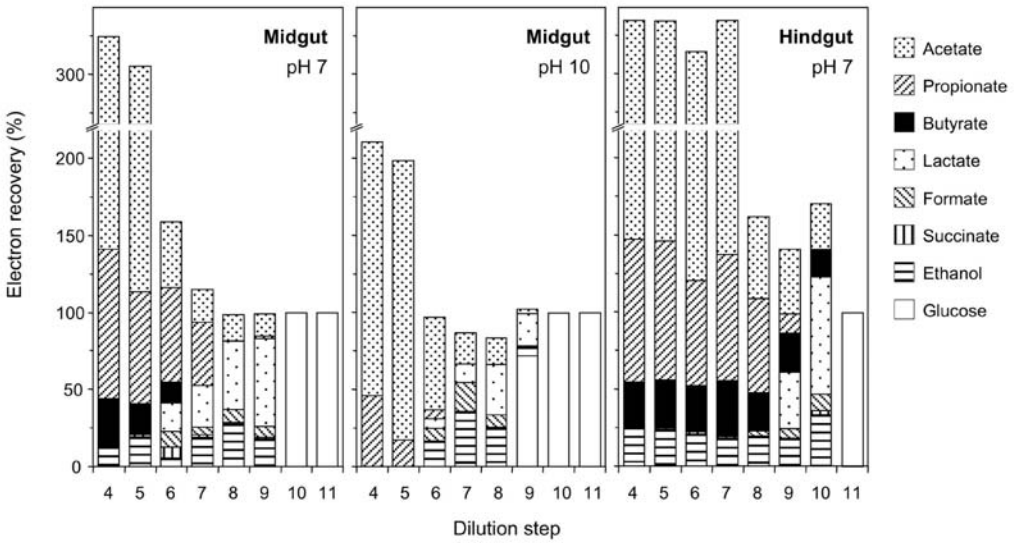


FIG. 3. Metabolic product profiles of enrichment cultures on glucose inoculated with 10-fold serial dilutions of midgut and hindgut homogenates of a *P. ephippiata* larva (third instar), incubated at pH 7 or 10. A dilution step of n is equivalent to 10^{-n} gut equivalents in the inoculum.

that of the hindgut, it has been pointed out already by several authors that the structure of the hindgut epithelium indicates that the proctodeal dilation is a zone of absorption also (2, 50, 60).

Major metabolic groups of microorganisms. The bacterial density in the two major gut compartments of *P. ephippiata* is similar to that reported for *P. marginata* (21). Little is known, however, about the microorganisms colonizing the intestinal tract of scarab beetle larvae and their metabolic activities.

TABLE 4. Abundance of major metabolic groups in serial dilutions of midgut and hindgut homogenates of *P. ephippiata* larvae (third instar)[a]

Metabolic group	Substrate	Midgut	Hindgut
Aerobic[b]	Cellulose[c]	8	7
	Glucose	9–10	10
	Casamino Acids[d]	9	10
	Vanillin, cinnamate	6–7	8
Fermenting[e]	Cellulose[c]	<4[f]	<4
	Glucose	9	10
	Casamino Acids[d]	8	9
	Lactate	7–8	8–9
Methanogenic[e]	H_2-CO_2	6–8	8–10
Homoacetogenic[e]	H_2-CO_2	4–5	7–8
Sulfate reducing[e]	Lactate, formate	4	6

[a] Values represent $\log_{10}$ of the last positive dilution in three to four replicate series at pH 7.
[b] Medium MM-5 incubated under air.
[c] Filter paper disks (equivalent to 5 mM glucose).
[d] Concentration was increased to 0.5 mg liter^{-1}.
[e] Medium AM-5 under H_2-CO_2 atmosphere.
[f] Dilution series started with the 10^4 dilution.

The cultivation-independent study published in the companion paper (28) showed that the microbial community in the midgut is composed almost exclusively of gram-positive bacteria, with clones associated with the *Actinobacteria*, the *Clostridiales*, and, to a lesser extent, the *Lactobacillales* and *Bacillales* dominating the clone library. Interestingly, several clones were virtually identical to the 16S rRNA sequence of *Promicromonospora pachnodae* isolated from the gut of *P. marginata* (23), which is closely related to the cellulolytic bacterium *Cellulosimicrobium variabile* isolated from the hindgut of the termite *Mastotermes darwiniensis* (1). Its presence in large numbers also in the gut of *P. ephippiata* larvae (O. Geissinger and A. Brune, unpublished results) suggests that *P. pachnodae* may be a specific member of the gut microbiota of *Pachnoda* larvae. *P. pachnodae*, initially designated "*Cellulomonas pachnodae*" (22), is a facultatively anaerobic bacterium that possesses endoglucanase and xylanase activity and ferments glucose to acetate, lactate, ethanol, and formate (23), which is in good agreement with the metabolic product pattern of the serial dilutions (Fig. 3).

The microbial community composition in the hindgut of *P. ephippiata*, however, differs markedly from that in the midgut (28). The clone library was dominated by clones associated with the *Cytophaga-Flavobacterium-Bacteroides* phylum, the *Clostridiales*, and the *Lactobacillales*; clones related to *Bacillales* and *Actinobacteria* were of minor frequency. Interestingly, the first bacterium ever isolated from scarab beetle larvae was a "*Bacillus cellulosam fermentans*" (59)—a gram-negative, obligately anaerobic, spore-forming bacterium that did not grow on monosaccharides, disaccharides, or starch, resembling Omelianski's "Wasserstoffbacillus" (45) and Khouvine's "*Bacillus cellulosae dissolvens*" (39) in the strong production of hydrogen and acidic fermentation products. The isolate was eventually

lost, but from the description and its association with cellulose fibers (59), it can be safely concluded that it was an obligate anaerobe, possibly representing a cellulolytic member of the *Cytophaga-Flavobacterium-Bacteroides* phylum or the clostridia.

Representatives of both groups seem to be numerically important in the gut of *P. ephippiata* (28). The presence of clostridia and *Bacteroides*-related bacteria is in good agreement with the formation of butyrate as a characteristic product in the highest dilutions of hindgut contents. Since butyrate production—at least in clostridia—is dependent on the hydrogen partial pressure in the environment (51), the apparent absence of butyrate accumulation in the hindgut fluid may be explained by the low H_2 partial pressure maintained in all hindgut regions.

Microorganisms capable of aerobic and fermentative metabolism appear to be similar in abundance or may even represent populations of facultatively anaerobic bacteria. Only the bacteria mineralizing vanillate and cinnamate seem to be obligately dependent on the influx of oxygen into the gut. Just as in the case of the termite gut microbiota (17), such lignin-derived aromatic compounds were not degraded under anoxic conditions; only demethylation of vanillate and side chain reduction of cinnamate occurred in the lower dilutions.

Methanogenesis. In *P. ephippiata*, methanogenesis takes place exclusively in the hindgut paunch but not in the alkaline midgut. This is in good agreement with the results of the cultivation-independent characterization of the archaeal community in the different gut compartments, which revealed that the dominant archaea in the midgut were (most likely nonmethanogenic) *Crenarchaeota* (28). Methanogenesis is restricted to the hindgut also in the larvae of other scarabaeid beetles (3, 31), and the methane emission rates are in the same range as those determined for *P. ephippiata* (Table 1). The large individual variance could be at least partly attributable to scarabaeid beetle larvae emitting CH_4 not by flatulence but by spiracular-controlled 6-min bursts only once every 1.5 h, as demonstrated for *Pachnoda butana* (6).

Similar to the situation in cockroaches and soil-feeding termites (42, 53), the methane emission rates of isolated hindguts of *P. ephippiata* were considerably lower than those of whole larvae, and methanogenesis was stimulated considerably by exogenous electron donors. In cockroaches (*Blaberus* sp.), a cross-epithelial transfer of H_2 from the midgut compartment drives methanogenesis in the hindgut (42), and also the high in vivo rates of methanogenesis in soil-feeding termites (*Cubitermes* spp.) have been explained by the proximity of H_2-producing and H_2-consuming gut compartments within the abdomen (53). However, in scarab beetle larvae, the discrepancy between methane emission rates of whole animals and isolated hindguts, which is also evident from the data for *Oryctes nasicornis* (3), cannot be explained by the same mechanism. H_2 does not accumulate in the midgut of *P. ephippiata*, and since the bulk of midgut and hindgut is not in direct contact in scarab beetle larvae (Fig. 1C), the highly diffusible H_2 molecule would not be a good transport form of reducing equivalents. Rather, the accumulation of formate as a major product of fermentation in the midgut, its presence in the hemolymph, and the strong stimulatory effect of formate on hindgut methanogenesis indicate that midgut fermentations are coupled to methanogenesis in the hindgut by formate transported via the hemolymph.

Conclusion. The digestive tract of humus-feeding scarab beetle larvae shows considerable parallels to the situation in soil-feeding termites, particularly with respect to the alkaline gut regions. The gut of *P. ephippiata* larvae harbors a dense and diverse microbiota, which differs considerably among the major gut regions and from that in the soil fed to the larvae (28). To date, only a few of these bacteria have been isolated in pure culture, but the results of the present study are quite encouraging for further cultivation-based investigations, which are important to improve our understanding of the functional interactions of the symbiotic microbiota involved in the digestion of soil organic matter by humivorous insects.

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3.2 Struktur mikrobieller Gemeinschaften in Mittel- und Enddarm humivorer Rosenkäferlarven

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Zusammenfassung: Vor dem Hintergrund, mehr über die Rolle der Darmflora bei der Umsetzung organischer Substanz durch Boden-Invertebraten zu erfahren, wurden die bakterielle und archaeelle Diversität in Futterboden, Mitteldarm und Enddarm humivorer Rosenkäferlarven (*Pachnoda ephippiata*, *Scarabaeidae*, *Coleoptera*) mit Hilfe molekularer Methoden (16S rRNA-Gen Klonbibliotheken und T-RFLP-Analyse) untersucht. Zuvor war in einer begleitenden Studie (Kapitel 3.1) gezeigt worden, dass der Mitteldarm dieser Larven extrem alkalisch ist, dass sich in Mittel- und Enddarm hohe Konzentrationen an Gärprodukten finden und dass beide Darmabschnitte dicht mit (unbekannten) Mikroorganismen besiedelt sind. Die molekulare Diversitätsanalyse zeigte deutliche Unterschiede in den mikrobiellen Gemeinschaften von Futterboden und Darm auf, ein Hinweis auf eine hohe Spezifität der Darmflora. Die mikrobielle Diversität war in Mitteldarm (dominiert von *Actinobacteria*) und Enddarm (dominiert von Vertretern des CFB-Phylums) sehr hoch; dass v.a. potentiell gärende Bakterien (z.B. *Lactobacillales*, *Clostridiales*, Vertreter des CFB-Phylums) detektiert wurden, passte gut zu den hohen Acetat- und Lactat-Konzentrationen sowie den hohen Titern lactogener und acetogener Bakterien, die in der Begleitstudie (Kapitel 3.1) gefunden wurden. Die archaeelle Diversität war deutlich geringer als die der Bakterien. Während im Mitteldarm mesophile Crenarchaeoten (relativ) dominierten, die allerdings sehr wahrscheinlich aus dem gefressenen Boden stammten, dominierten im Enddarm *Methanobrevibacter*-Arten. Letzteres erklärt, warum die Methanogenese bei *Pachnoda*-Larven auf den Enddarm beschränkt ist.

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Microbial Community Structure in Midgut and Hindgut of the Humus-Feeding Larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae)

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The guts of soil-feeding macroinvertebrates contain a complex microbial community that is involved in the transformation of ingested soil organic matter. In a companion paper (T. Lemke, U. Stingl, M. Egert, M. W. Friedrich, and A. Brune, Appl. Environ. Microbiol. 69:6650–6658, 2003), we show that the gut of our model organism, the humivorous larva of the cetoniid beetle *Pachnoda ephippiata*, is characterized by strong midgut alkalinity, high concentrations of microbial fermentation products, and the presence of a diverse, yet unstudied microbial community. Here, we report on the community structure of bacteria and archaea in the midgut, hindgut, and food soil of *P. ephippiata* larvae, determined with cultivation-independent techniques. Clone libraries and terminal restriction fragment length polymorphism analysis of 16S rRNA genes revealed that the intestines of *P. ephippiata* larvae contain a complex gut microbiota that differs markedly between midgut and hindgut and that is clearly distinct from the microbiota in the food soil. The bacterial community is dominated by phylogenetic groups with a fermentative metabolism (*Lactobacillales*, *Clostridiales*, *Bacillales*, and *Cytophaga-Flavobacterium-Bacteroides* [CFB] phylum), which is corroborated by high lactate and acetate concentrations in the midgut and hindgut and by the large numbers of lactogenic and acetogenic bacteria in both gut compartments reported in the companion paper. Based on 16S rRNA gene frequencies, *Actinobacteria* dominate the alkaline midgut, while the hindgut is dominated by members of the CFB phylum. The archaeal community, however, is less diverse. 16S rRNA genes affiliated with mesophilic *Crenarchaeota*, probably stemming from the ingested soil, were most frequent in the midgut, whereas *Methanobacteriaceae*-related 16S rRNA genes were most frequent in the hindgut. These findings agree with the reported restriction of methanogenesis to the hindgut of *Pachnoda* larvae.

Saprophagous macroinvertebrates, such as earthworms, termites, and many coleopteran and dipteran larvae, play a major role in the degradation and stabilization of soil organic matter (SOM) and strongly influence important physical and chemical soil parameters (34, 61). The transformation of SOM during passage through the alkaline guts of humivorous insects has so far been investigated only with soil-feeding termites (for reviews see references 5, 6, and 8). It has been shown that the conditions in the anterior hindgut of *Cubitermes* spp. (high alkalinity and oxygen influx) enhance the extraction of organic matter from the inorganic matrix, cause chemical oxidation of humic substances, and lead to a decrease of the molecular weight of the organic matter (30). The resulting increase in solubility renders the organic matter accessible for digestion in subsequent, less-alkaline compartments (27, 28, 31). Although the complex microbial community in the guts of humivorous macroinvertebrates is believed to participate in the transformation of ingested SOM (10, 29), detailed information on the composition and activities of the gut microbiota is lacking.

To elucidate the contribution of gut microorganisms to digestion of SOM and host nutrition, information about structure and function of the microbial gut community has to be linked to gut morphology, the physicochemical conditions in different gut compartments, and the spatial distribution of the microorganisms (8, 9). Due to difficulties in simulating the microenvironment of an insect gut system, which is characterized by steep and overlapping O_2, H_2, pH, and redox gradients (8), culture-based approaches do not adequately reflect the microbial diversity in the guts of soil feeders, and it is necessary to study the structure of microbial gut communities with culture-independent techniques (2). To date, such studies have been restricted to only a few arthropods, mostly termites (7, 19, 25, 32, 37, 44, 45, 53), where especially the combination of clonal and terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes has provided a deeper insight into the gut microbial community of soil-feeding termites and its dependence on axial gut differentiation (19, 51, 52).

The gut of our model organism, the humivorous larva of the cetoniid beetle *Pachnoda ephippiata* (Coleoptera: Scarabaeidae), resembles the termite gut with respect to strong midgut alkalinity, high concentrations of microbial fermentation products, and methane emission from the hindgut (see the companion paper [35]). Like the termite gut, the *Pachnoda* gut is also characterized by a dense and diverse microbial community (11, 35), which is obviously involved in digestion, but whose exact composition and role remain to be studied.

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In order to gain more insight into the role of the gut microbiota of scarab beetles in the transformation of SOM, we used a culture-independent approach, combining a clonal analysis and T-RFLP fingerprinting analysis of 16S rRNA genes, to investigate the bacterial and archaeal community structure in midgut and hindgut of *P. ephippiata* larvae and to compare it to that in the soil consumed by the larvae.

MATERIALS AND METHODS

DNA extraction and purification. Larvae of *P. ephippiata* (second instars) were taken from a controlled breeding facility at the University of Konstanz and dissected as described in the companion paper (35). Guts were separated into midgut and hindgut and stored frozen at −20°C until further analysis. To minimize the influence of differences between individual larvae, DNA was extracted from four pooled gut sections each and food soil (0.5 g), according to a direct lysis protocol that has been previously described in detail (23). DNA was purified from the supernatant by consecutive ammonium acetate, isopropanol, and ethanol precipitation steps. To remove humic substances, extracts were passed through spin columns filled with polyvinylpolypyrrolidone as described previously (48). The protocol was further improved by using a swinging bucket rotor (A-8-11 for Eppendorf centrifuge 5417 R; Eppendorf, Hamburg, Germany) so that the amount of polyvinylpolypyrrolidone suspension necessary for sufficient purification could be reduced to ≤500 μl. Extraction efficiency and quality of extracted DNA were verified by standard gel electrophoresis.

PCR amplification of bacterial and archaeal 16S rRNA genes. 16S rRNA genes were specifically amplified from gut DNA extracts with the primer combination 27f (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) (17) and 1492r (5′-TAC-GGY-TAC-CTT-GTT-ACG-ACT-T-3′) (33) targeting *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) (20) and Ar912r (5′-CTC-CCC-CGC-CAA-TTC-CTT-TA-3′) (40) targeting *Archaea*. The reaction mixture contained, in a total volume of 50 μl, 1× PCR buffer II (Applied Biosystems, Weiterstadt, Germany), 1.5 mM $MgCl_2$, 50 μM concentrations of each of the four deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 μM concentrations of each primer (MWG Biotech, Ebersberg, Germany), 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems), and 1 μl of a 1:30 dilution of the gut DNA extract. All reaction mixtures were prepared at 4°C in 0.2-ml reaction tubes to avoid nonspecific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94°C) block of a GeneAmp 9700 Thermocycler (Applied Biosystems). The standard thermal profiles for the amplification of 16S rRNA genes were as follows: initial denaturation (94°C, 3 min); 32 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 60 s) for *Bacteria*; and 35 cycles of denaturation (94°C, 45 s), annealing (52°C, 45 s), and extension (72°C, 90 s) for *Archaea*. After terminal extension (72°C, 5 to 7 min), samples were stored at 4°C until further analysis. Aliquots (5 μl) of 16S rRNA gene amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

16S rRNA gene libraries. Bacterial and archaeal 16S rRNA gene amplicons were cloned in *Escherichia coli* JM109 with the pGEM-T Easy Vector system (Promega, Mannheim, Germany) according to the manufacturer's instructions. Randomly selected clones were checked for correct insert size via standard vector-targeted PCR and gel electrophoresis. DNA was sequenced on an ABI Prism 377 DNA sequencer with Big Dye terminator chemistry as specified by the manufacturer (Applied Biosystems). Bacterial clones were designated PeM (midgut section; 60 clones) and PeH (hindgut section; 53 clones), and archaeal clones were designated PeMAr (midgut section; 24 clones) and PeHAr (hindgut section; 44 clones). Clones from food soil (only *Archaea*) were named FSAr (24 clones).

T-RFLP analysis. For T-RFLP analysis, 16S rRNA genes were PCR amplified as described above, except that for bacterial clones, 6-carboxyfluorescein-labeled 27f forward primer, nonlabeled 907r (5′-CCG-TCA-ATT-CCT-TTR-AGT-TT-3′) (42) backward primer, and an annealing temperature of 52°C were used. For archaeal clones, primer Ar912r was 6-carboxyfluorescein labeled. DNA concentrations in the PCR products were determined photometrically. For digestion, ~75 ng of DNA, 2.5 U of restriction enzyme (*Msp*I for *Bacteria* and *Taq*I or *Alu*I for *Archaea*; Promega), 1 μl of the appropriate 10× incubation buffer (Promega), and 1 μg of bovine serum albumin were combined in a total volume of 10 μl and digested for 3 h at 65°C (*Taq*I) or 37°C (*Msp*I and *Alu*I). Fluorescently labeled terminal restriction fragments (T-RFs) were size separated on an ABI 373A automated sequencer (Applied Biosystems) with an internal size standard (GeneScan-1000 ROX; Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems). To determine the relative 16S rRNA gene frequency, the fluorescence intensity of each individual peak, i.e., a single T-RF, expressed as single peak height, was compared to the total fluorescence intensity of all T-RF peaks, expressed as total peak height.

Phylogenetic analysis. Sequence data were analyzed and trees were constructed by using the ARB software package with its database (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München, Munich, Germany; http://www.arb-home.de). 16S rRNA gene sequences were added to the database and aligned with the Fast Aligner tool (version 1.03). Alignments were corrected manually if necessary. All clonal 16S rRNA gene sequences were compared to sequences in public databases by using BLAST (1), and closely related sequences from databases were retrieved and added to the alignment. Trees were constructed using the neighbor-joining algorithm (50) and base frequency filters (50 to 100% similarity) for *Bacteria* or *Archaea* provided with the ARB package. For bacterial and archaeal tree construction, 1,198 sequence positions (*E. coli* numbering 59 to 1454) and 659 sequence positions (*E. coli* numbering 109 to 894), respectively, were used. Sequences of species closely related to the gut clones but significantly shorter than 1,500 bp (*Bacteria*) or 900 bp (*Archaea*) were added to the tree by using the ARB parsimony tool, which allows the addition of short sequences to phylogenetic trees without changing global tree topologies (39). The terminal sequence positions at the 5′ and 3′ ends of the 16S rRNA gene sequences (450 bp for archaeal clones and 750 bp for bacterial clones) were also subjected to a separate treeing analysis ("fractional treeing" [38]); significant differences in the phylogenetic placement of a fragment pair were considered indicative of chimera formation, and chimeric rRNA gene clones were excluded from further phylogenetic analysis.

Ecological indices. Bacterial and archaeal species richness in midgut and hindgut clone libraries was estimated using Chao1 (13) as a nonparametric indicator, calculated with EstimateS (version 5.0.1; R. Colwell, University of Connecticut [http://viceroy.eeb.uconn.edu/estimates]) as described in reference 26. For this purpose, a "species" was defined as a group of 16S rRNA gene clones with ≥97% sequence similarity (55). To determine the similarity of the compositions of different microbial communities based on T-RFLP analysis, Morisita community similarity indices (I_M) were calculated as described by Dollhopf et al. (16). For this purpose, only T-RFs with a relative peak height of ≥1% of the total electropherogram peak height were used. I_M values range from 0 to 1, with 1 indicating complete identity of the two compared communities.

Nucleotide sequence accession number. 16S rRNA gene sequences of clones from the gut of *P. ephippiata* larvae and food soil are accessible under numbers AJ576118 to AJ576204 (archaeal clones) and AJ576323 to AJ576428 (bacterial clones). Clones PeM75, PeH59, and PeMAr04 are accessible under numbers AJ538350 to AJ538352, respectively.

RESULTS

16S rRNA gene clone libraries. Four separate clone libraries were generated from the 16S rRNA gene fragments amplified from midgut and hindgut DNA with *Bacteria*-specific and *Archaea*-specific primer pairs, respectively. An additional archaeal clone library was generated using the DNA extracted from the food soil.

From the bacterial clone libraries, randomly selected clones from the midgut (60 clones) and hindgut (53 clones) were sequenced. Phylogenetic analysis revealed that most clones were affiliated with known taxa of the *Bacteria*. Four midgut clones and one hindgut clone were identified as chimeras and were excluded from further analysis.

The remaining 56 clones from the midgut clone library could be assigned to 10 distinct phylogenetic groups (Fig. 1 and 2). Most clones (36%) were affiliated with *Actinobacteria*, followed by clones related to *Clostridiales*, *Lactobacillales*, and *Bacillales* (Table 1). Six midgut clones were closely related (96% sequence similarity) to *Turicibacter sanguinis*, a recently described strictly anaerobic gram-positive bacterium isolated from a blood sample of a human with acute appendicitis (4). Some of the midgut clones clustered with *Proteobacteria* from

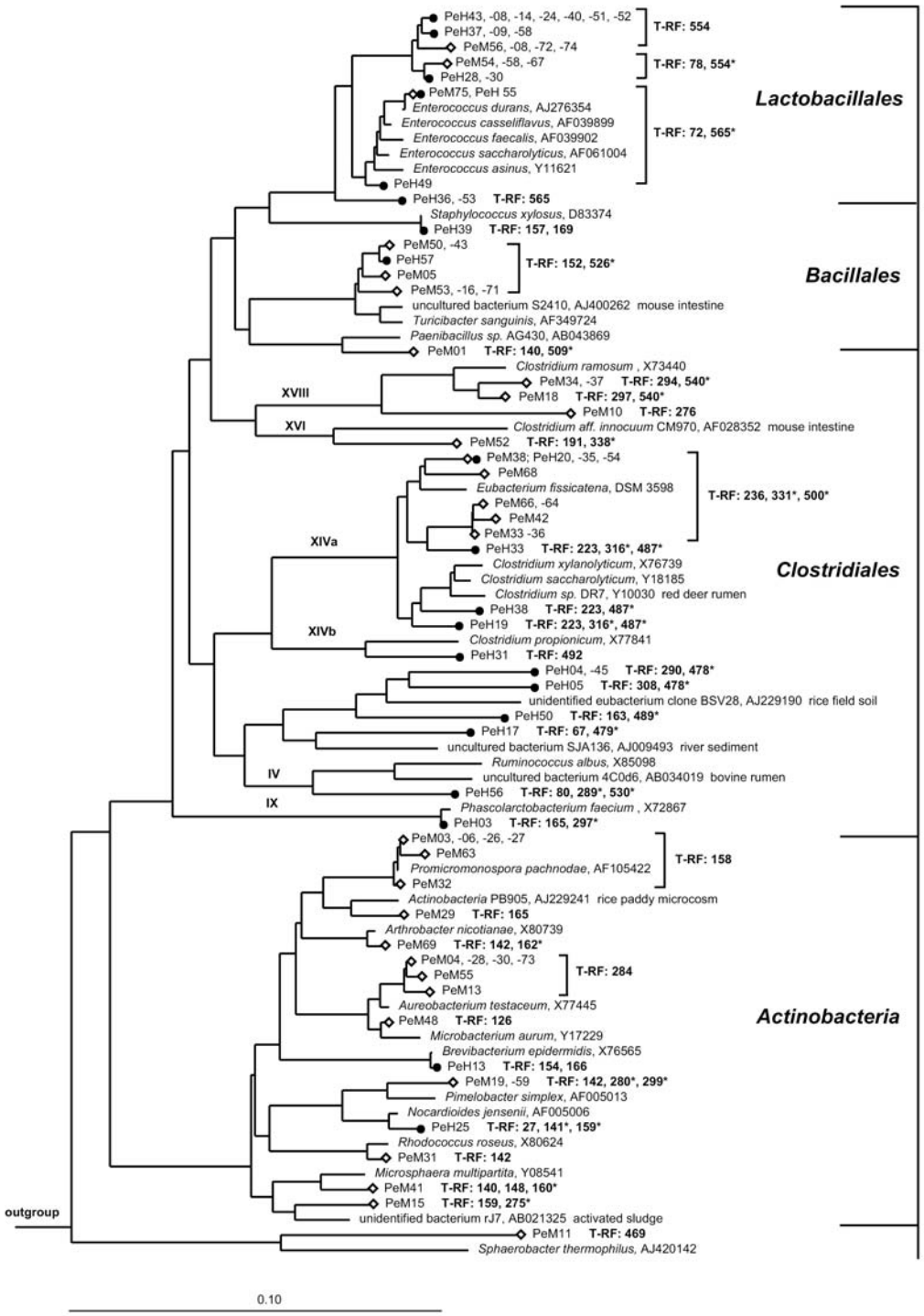

FIG. 1. Phylogenetic tree showing the positions of bacterial 16S rRNA gene sequences affiliated with gram-positive bacteria, recovered fro the midgut (♢) and hindgut (●) of *P. ephippiata* larvae. Scale bar represents 10% sequence difference. Accession numbers of reference sequenc are indicated. Species used as the outgroup were *Thermus thermophilus* (M26923), *Fervidobacterium gondwanense* (Z49117), and *Thermoto maritima* (M21774). Roman numerals indicate clostridial subgroups sensu (14). Lengths of T-RFs result from in vitro digestion of clonal 16S rRN gene amplicons with *Msp*I; pseudo-T-RFs are marked with asterisks.

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TABLE 1. Relative abundance (percent) of major phylogenetic groups in the midgut and hindgut of *P. ephippiata* larvae, based on the frequencies of 16S rRNA genes in 16S rRNA gene clone libraries and on T-RFLP analysis[a]

Phylogenetic group	Midgut		Hindgut	
	Clone library	T-RFLP	Clone library	T-RFLP
Actinobacteria	35.7	36.9–64.0	3.8	2.0–10.4
Bacillales	12.5	9.4–28.1	3.8	1.3–5.9
Lactobacillales	14.3	7.0	30.8	16.4–20.9
Clostridiales	21.4	5.4–9.1	26.9	21.8–28.9
CFB phylum	1.8	1.7	26.9	33.7–44.0
Planctomycetales	3.6	0–7.3	ND	ND
β-*Proteobacteria*	3.6	0–2.1	3.8	0–4.5
γ-*Proteobacteria*	1.8	0–0.5	ND	ND
δ-*Proteobacteria*	1.8	NA	1.9	NA
ε-*Proteobacteria*	ND	ND	1.9	NA
Sphaerobacter related	1.8	NA	ND	ND
TM7 phylum	1.8	NA	ND	ND

[a] For an explanation of the T-RFLP-based frequency ranges, see the text. ND, not detected; NA, not assignable.

the β, γ, and δ subdivisions; clones related to members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum, *Planctomycetales*, and *Sphaerobacter thermophilus* were rare (Table 1). Clone PeM47, not displayed in the figures, grouped with a human mouth clone (BS003) from the TM7 phylum (46). The 52 hindgut clones were mostly affiliated with *Lactobacillales*, *Clostridiales*, and members of the CFB phylum (Fig. 1 and 2). Clones related to *Proteobacteria* (β, δ, and ε subdivisions), *Actinobacteria*, and *Bacillales* were recovered less frequently (Table 1).

By using an arbitrarily defined limit of 97% sequence similarity, the 16S rRNA gene clones in the bacterial midgut and hindgut clone libraries can be grouped into 29 or 33 different species, respectively. The total species richness of the midgut and hindgut communities was calculated using the data from the clone libraries and Chao1 as a nonparametric richness estimator (26). The estimated numbers of bacterial species were 96 for the midgut and 118 for the hindgut community, which means that the clonal approach detected about one-third of bacterial species actually present in the midgut and

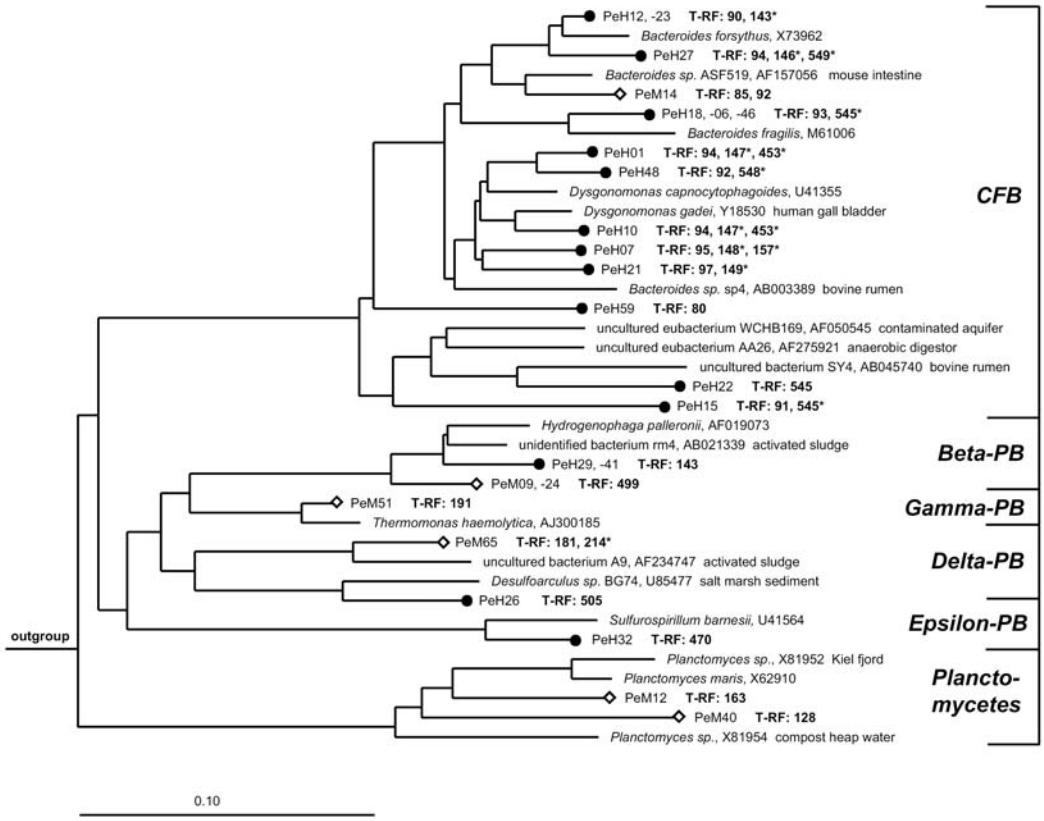

FIG. 2. Phylogenetic tree showing the positions of bacterial 16S rRNA gene sequences affiliated with the CFB phylum, the *Proteobacteria* (PB), and the *Planctomycetales*, recovered from the midgut (◇) and hindgut (●) of *P. ephippiata* larvae. Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. Species used as the outgroup were *Thermus thermophilus* (M26923), *Fervidobacterium gondwanense* (Z49117), and *Thermotoga maritima* (M21774). Lengths of T-RFs result from in vitro digestion of clonal 16S rRNA gene amplicons with *Msp*I; pseudo-T-RFs are marked with asterisks.

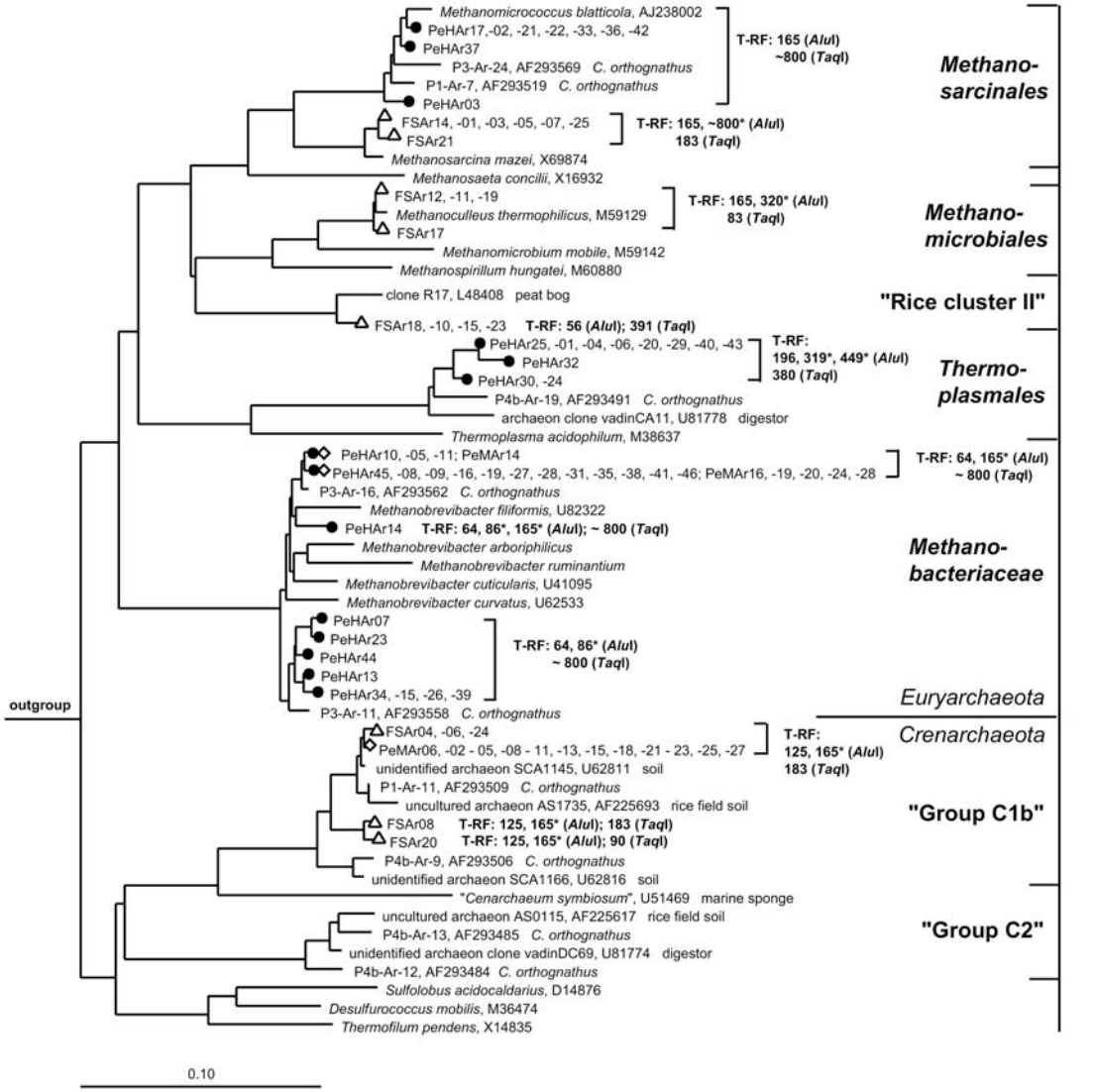

FIG. 3. Phylogenetic tree showing the positions of archaeal 16S rRNA gene sequences affiliated with *Euryarchaeota* and *Crenarchaeota*, recovered from the midgut (◇) and hindgut (●) of *P. ephippiata* larvae and from the soil fed to the larvae (△). Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. Species used as outgroup were *Nitrospira marina* (X82559), *Chloroflexus aurantiacus* (M34116), *Holophaga foetida* (X77215), *Rhodothermus marinus* (X77140), *Streptomyces coelicolor* (X60514), and *Acidobacterium capsulatum* (D26171). Lengths of T-RFs result from in vitro digestion of clonal 16S rRNA gene amplicons with *Alu*I or *Taq*I; pseudo-T-RFs are marked with asterisks.

hindgut communities. Since the corresponding 95% confidence intervals were 57 and 186 for the midgut community and 72 and 214 for the hindgut community, species richness in the hindgut is not significantly higher than that in the midgut (26).

For the archaeal clone libraries, 30 randomly selected clones from the midgut, 45 clones from the hindgut, and 24 clones from the food soil were sequenced. Phylogenetic analysis revealed no chimera, but six midgut clones, one hindgut clone, and four soil clones were not affiliated with the *Archaea* and were excluded from further analyses. The remaining clones were all affiliated with known taxa of *Euryarchaeota* and *Crenarchaeota* (Fig. 3). Seventy-five percent of the midgut clones were affiliated with mesophilic *Crenarchaeota* (group C1b sensu DeLong and Pace [15]), and the remaining clones clustered with the *Methanobacteriaceae* (Fig. 3). In contrast, the hindgut library was dominated by *Methanobacteriaceae*-related

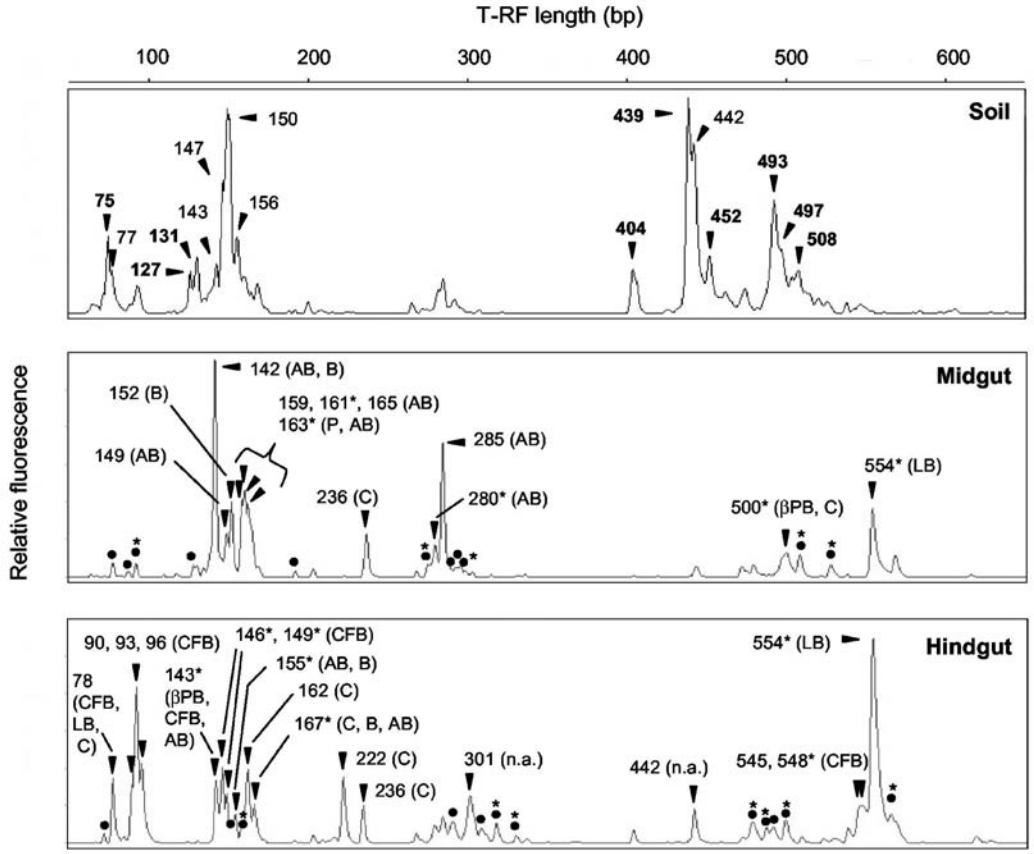

FIG. 4. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts of the midgut and hindgut of *P. ephippiata* larvae and food soil. *Msp*I was used for restriction digestion. All major T-RFs (≥2% of total peak height; triangles), assignable minor T-RFs (dots), and peaks probably influenced by pseudo-T-RF formation (asterisks) are marked. Assignable phylogenetic groups are given in parentheses with the following abbreviations: AB, *Actinobacteria*; B, *Bacillales*; CFB, CFB phylum; C, *Clostridiales*, LB, *Lactobacillales*; P, *Planctomycetales*; βPB, β-*Proteobacteria*; n.a., not assignable. Boldface T-RFs in the soil profiles indicate major T-RFs without a corresponding major T-RF in the gut profiles.

clones (55%), and the remaining clones were affiliated almost in equal shares with *Thermoplasmales* and *Methanosarcinales*. The clones related to the *Euryarchaeota* clustered with methanogenic species isolated from other insect guts, such as *Methanomicrococcus blatticola* (96 to 97% sequence similarity), *Methanobrevibacter filiformis* (96% similarity), and *Methanobrevibacter curvatus* (95 to 96% similarity). Moreover, all archaeal clones obtained from the *P. ephippiata* gut clustered with clones obtained from the hindgut of *Cubitermes orthognathus*, a soil-feeding termite (19).

Using a 97% sequence similarity threshold, the archaeal midgut clone library and the hindgut clone library contained two and five different species, respectively. Species richness estimation with Chao1 yielded the same result, and 95% confidence intervals of zero provided strong evidence that all archaeal species from the *P. ephippiata* intestine detectable with the applied primer system were represented in the clone library and that archaeal species richness in the hindgut can be regarded as being significantly higher than that in the midgut.

Thirty-five percent of the soil clones were affiliated with members of the *Methanosarcinales*, but in contrast to the gut clones, they were closely related to *Methanosarcina thermophila*. Four soil clones each (20%) were affiliated with the *Methanomicrobiales* and uncultured archaea from rice field soil (rice cluster II [21]). Five soil clones (25%) were affiliated with mesophilic *Crenarchaeota* and clustered with the clones obtained from the *P. ephippiata* and *C. orthognathus* guts.

T-RFLP analyses. Structure and diversity of the microbial communities in the two *P. ephippiata* gut sections and in the food soil were also assessed directly by T-RFLP analysis of bacterial and archaeal 16S rRNA gene fragments. In silico determination of the expected size of T-RFs allowed us to assign T-RFs in the fingerprints of gut and soil communities to distinct phylogenetic groups (Fig. 4 and 5). In the context of this study, we observed that several of the clones exhibited additional peaks in the T-RFLP analysis, which we called "pseudo-T-RFs" (18). Pseudo-T-RFs are restriction fragments longer than the expected T-RF of a clone and form an addi-

tional, albeit smaller, peak in the T-RFLP profile. They are caused by partially single-stranded 16S rRNA gene amplicons, formed during the PCR, where the terminal restriction site is not accessible for the restriction endonucleases (18). Therefore, all clones were tested for their in vitro T-RF formation pattern to detect those showing pseudo-T-RF formation and to interpret the corresponding peaks in the community T-RFLP profiles (Fig. 1 to 3).

Since many T-RFs were shared by more than one phylogenetic group (Fig. 4 and 5), the frequency of certain phylogenetic groups in the *P. ephippiata* gut, when based on T-RFLP data, can be expressed only as frequency ranges (Table 1). Minimum frequencies were calculated by considering only those T-RFs that were unique for a certain group, whereas for maximum frequencies those T-RFs shared with others were also taken into account.

In the T-RFLP profile of bacterial 16S rRNA genes from the midgut, all major T-RFs (≥2% of total peak height) and 12 out of 23 minor T-RFs (<2% of total peak height) could be assigned to phylogenetic groups from the midgut clone library (Fig. 4). In total, the peaks assigned to clones represented 90% of the total peak height. All phylogenetic groups in the midgut clone library, except those affiliated with the δ-*Proteobacteria*, *S. thermophilus*, and the TM7 phylum, were represented by T-RFs in the electropherogram. In the T-RFLP profile of bacterial 16S rRNA genes from the hindgut sample, 15 out of 17 major and 12 out of 18 minor T-RFs were assignable to phylogenetic groups from the hindgut clone library (Fig. 4). Eighty-eight percent of the total peak height could be assigned, and all phylogenetic groups from the clone library except δ-*Proteobacteria* and ε-*Proteobacteria* were represented by T-RFs.

16S rRNA gene frequencies estimated from the T-RFLP profiles (Table 1) generally corroborated the results obtained with the clone libraries. Differences were found with respect to the frequencies of 16S rRNA genes related to *Clostridiales* and *Lactobacillales*, which seem to be overrepresented in the midgut clone library, whereas those of *Actinobacteria* were apparently underrepresented. Also in the hindgut clone library, 16S rRNA genes related to *Lactobacillales* appeared to be overrepresented compared to the T-RFLP data, whereas genes related to the CFB phylum were apparently underrepresented. The Morisita index of community similarity (I_M) was 0.40 for the comparison of midgut with hindgut, underlining the clear differences in the bacterial community composition between the major gut sections; this difference was also displayed by the clone libraries.

As some archaeal clones were lacking *Taq*I restriction sites, T-RFLP analysis of archaeal community structure was performed also with *Alu*I to resolve these peaks further. In the T-RFLP profiles of the midgut, all major T-RFs (≥5% of total peak height) could be assigned to clones in the archaeal clone library of the midgut, and for all phylogenetic groups from the clone library, the corresponding T-RFs were detected (Fig. 5). In total, 94 and 91% of the total electropherogram peak height obtained using *Taq*I or *Alu*I, respectively, could be assigned to clones. Also in the archaeal T-RFLP profiles of the hindgut, all major T-RFs, representing 99% (*Taq*I) or 95% (*Alu*I) of total peak height, could be assigned to clones (Fig. 5).

The results of the T-RFLP analysis also affirmed the clone

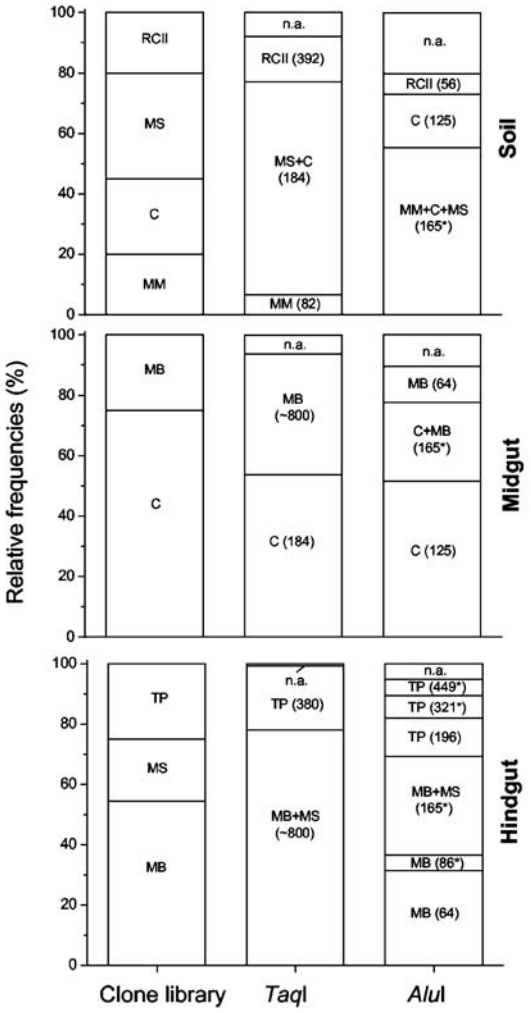

FIG. 5. Relative frequencies of distinct archaeal groups in the midgut, hindgut, and food soil of *P. ephippiata* larvae, based on composition of 16S rRNA gene clone libraries and T-RFLP analysis, with *Taq*I and *Alu*I. T-RF frequencies were calculated by comparing the individual heights of assignable T-RFs to the sum of all peak heights in the electropherograms. T-RF lengths in base pairs are given in parentheses; T-RFs affected by formation of pseudo-T-RFs are marked with an asterisk (see text). C, *Crenarchaeota*; MB, *Methanobacteriaceae*; MM, *Methanomicrobiales*; MS, *Methanosarcinales*; RCII, rice cluster II; TP, *Thermoplasmales*, n.a., not assignable.

frequencies observed in the clone libraries (Fig. 5). 16S rRNA genes of *Crenarchaeota*, which have the highest relative abundance in the midgut, were also restricted to this section, whereas those affiliated with *Thermoplasmales* were located exclusively in the hindgut. Clones closely related to *Methanomicrococcus blatticola* (no *Taq*I restriction site, 165-bp T-RF with *Alu*I) were restricted to the hindgut, as shown by their lack of appearance in the midgut clone library and by the 165-bp

T-RF in the midgut profile proving to be a pseudo-T-RF caused by clones related to *Crenarchaeota* and *Methanobacteriaceae* (18). An I_M value of 0.48 (calculated for *Alu*I-derived T-RF patterns) underlines the idea that the *Pachnoda* midgut and hindgut differ considerably in their archaeal community composition.

The relative gene frequencies deduced from the results of the T-RFLP analysis of the hindgut DNA were in good accordance with the 16S rRNA gene frequencies in the hindgut clone library, whereas the clones related to *Crenarchaeota* appeared to be slightly overrepresented in the midgut library.

T-RFLP profiles of bacterial 16S rRNA genes of the food soil extract (Fig. 4) differed clearly from the gut profiles. Morisita indices of community similarity (I_M) were 0.28 for the comparison of soil and midgut and 0.33 for the comparison of soil and hindgut. Therefore, no bacterial 16S rRNA gene clone library was created from the soil DNA extract. However, T-RFLP profiles of archaeal 16S rRNA genes showed relatively high similarities between food soil and midgut ($I_M = 0.69$) and food soil and hindgut ($I_M = 0.61$), particularly with respect to the occurrence of T-RFs of 183 bp (*Taq*I) and 125 and 165 bp (*Alu*I). Since these T-RFs all represent *Crenarchaeota*, the latter appear to be the only archaeal group occurring both in the soil and in the (mid)gut. The clone library data indicate an increase in the proportion of this group among the archaea from the soil (25%) to the midgut (75%); unfortunately, T-RFLP data cannot be used to corroborate this observation since all *Crenarchaeota* clones shared T-RFs with other archaeal clones. However, the T-RFLP profiles clearly document that 16S rRNA genes related to *Methanomicrobiaceae* and rice cluster II are both restricted to the soil. T-RFLP profiles of the gut samples lacked the corresponding *Taq*I T-RFs (82 and 392 bp [Fig. 5]). Clones related to *Methanobacteriaceae* and *Thermoplasmales* were found only in the gut samples. Neither their corresponding *Taq*I (~800 and 380 bp, respectively) nor *Alu*I (64, 196, 321, and 449 bp, respectively) T-RFs occurred in the soil electropherograms. As the 165-bp *Alu*I T-RF in the midgut electropherogram was a pseudo-T-RF (18), it can be excluded that *Methanosarcinales* species from the soil occur in the midgut. It can also be excluded that the *Methanosarcinales* present in the hindgut are ingested with the food soil since there was no ~800-bp T-RF in the soil electropherogram.

DISCUSSION

This is the first report on the intestinal microbiota of a beetle larva analyzed with cultivation-independent techniques. Our results showed that the morphological and physicochemical heterogeneity of the gut, described in the companion paper (35), is reflected in a high microbial diversity and pronounced axial differences in the composition of the gut microbiota, which is also clearly different from that in the soil fed to the larvae. Because in highly complex microbial communities a cloning approach is prone to the risk of undersampling (51), we employed T-RFLP analysis to corroborate the results of the 16S rRNA gene clone libraries and to document differences in community structures among the investigated samples with the necessary resolution.

Bacterial community. The bacterial community in the intestine of *P. ephippiata* larvae is highly diverse, which is typical for any gut system studied so far with culture-independent techniques (e.g., references 25, 36, 45, 56, and 60). Most clones obtained from the midgut and hindgut were affiliated with well-described phylogenetic groups commonly found in other intestinal tracts (e.g., *Lactobacillales*, *Bacillales*, *Clostridiales*, CFB phylum, and *Proteobacteria*; see literature cited above and references 8 and 9 for reviews about termites).

However, except for the clones related to *Promicromonospora pachnodae* (see below), only one clone obtained in this study clustered with clones or isolates from other arthropod guts, e.g., from termites (see studies cited in references 9 and 25). Clone PeH05, affiliated with the *Clostridiales*, was closely related (94% sequence similarity) to clone Rs-B65, obtained from the gut of the wood-feeding termite *Reticulitermes speratus* (25). Clones related to spirochetes, possibly accounting for up to 50% of all prokaryotes in some termites (e.g., references 25 and 47), were not detected at all, not even with a specific primer system (37) (data not shown). By contrast, the closest relatives of several clones stemmed from the intestines of higher animals (ruminants and rodents) or from human specimens, i.e., *Dysgonomonas* species (24) (90 to 95% sequence similarity) and *T. sanguinis* (96% similarity) (4). Obviously the gut microbiota of *P. ephippiata* larvae differs clearly from that of other soil arthropods, such as termites.

The apparent dominance of fermenting bacteria (*Lactobacillales*, *Clostridiales*, members of the CFB phylum, and clones related to *T. sanguinis*) in the gut of *Pachnoda* larvae corroborates the high lactate and acetate concentrations in midgut and hindgut and the large numbers of lactogenic and acetogenic bacteria in both compartments (35). The dramatic changes in the physicochemical conditions between the midgut and hindgut of scarabaeid beetle larvae (35) are in good agreement with the major differences in the bacterial community structure: the relative dominance of 16S rRNA genes of *Actinobacteria* in the midgut and of members of the CFB phylum in the hindgut. The combined results of clonal and T-RFLP analysis also document that *Bacillales*, *Planctomycetales*, and γ-*Proteobacteria* occur mainly in the midgut, whereas β-*Proteobacteria* are present in the midgut and hindgut. As T-RFLP analysis revealed no T-RFs corresponding to clones affiliated with the δ and ε subgroups of *Proteobacteria*, the TM7 phylum, and *S. thermophilus*, these groups are obviously overrepresented in the clone libraries, which contain only a limited number of clones. Also the high frequency of *Clostridiales*-related 16S rRNA genes in the midgut library was not supported by the T-RFLP profiles, whereas the high proportion of *Clostridiales* in the hindgut and the twofold increase in frequency of *Lactobacillales* between midgut and hindgut were corroborated by both methods.

The most prominent among the environmental factors possibly determining major differences in bacterial community structure between the midgut and hindgut of *P. ephippiata* larvae are the availability of oxygen and the intestinal pH. Although both compartments are largely anoxic, the influx of oxygen via the gut epithelium should be considerably larger in the case of the midgut because of its tubular shape (35). This might explain why many of the midgut clones grouped among *Actinobacteria*, which are capable of aerobic metabolism, and why many of the hindgut clones were affiliated with *Bacteroides*

species or the *Clostridiales*, whose members are known as obligate anaerobes.

In view of the high midgut alkalinity, it is reasonable to assume that at least some of the bacteria in the midgut are alkaliphilic. One clone (PeM01) was distantly related (92%) to an alkaliphilic *Bacillus* species that was isolated from soil (43). Six midgut clones were closely related (>98%) to *P. pachnodae*, a facultatively aerobic, hemicellulolytic bacterium isolated from the hindgut of *Pachnoda marginata* larvae (12). Interestingly, all *Promicromonospora*-related clones obtained in the present study were recovered exclusively from the *P. ephippiata* midgut, as were the vast majority of the clones representing *Actinobacteria*. However, it is not possible to infer the pH tolerance of the bacteria represented by the clones from the phylogenetic analysis, but it is tempting to speculate that bacilli and *Actinobacteria* might participate in the hydrolysis of polysaccharides in this gut compartment.

Archaeal community. Our data document clear differences between the archaeal communities in the midgut and hindgut of *P. ephippiata* larvae, which were also less diverse than the bacterial communities in the respective gut sections. In contrast to the bacterial clones, all archaeal clones clustered with clones or isolates previously retrieved from the guts of other insects, in particular from the soil-feeding termite *C. orthognathus* (19). As in the case of this termite, also the different archaeal populations in the gut of *P. ephippiata* larvae are highly specific for the respective gut compartment: while *Crenarchaeota*-like clones were absent from the hindgut, no clones affiliated with *Methanosarcinales* and *Thermoplasmales* were recovered from the midgut. Also the clones affiliated with *Methanobacteriaceae* and *Methanosarcinaceae* were more frequent in the hindgut. The apparent dominance of methanogenic archaea in the hindgut agrees with the restriction of methanogenesis to this compartment of *Pachnoda* larvae (22, 35). The absence of methane emission from the midgut indicates either that the absolute number of methanogens in this section is very small and/or that they are inactive. Cultivation-based enumeration showed that the numbers of methanogens in the midgut are about 2 orders of magnitude lower than those in the hindgut (35). It is likely that the presence of methanogens (and possibly also of other hindgut bacteria) in the midgut is merely the result of a reflux of hindgut content into the midgut, which has been described as a part of the digestion process in scarabaeid beetle larvae (49).

The close relationship of our clones to *Methanobrevibacter* and *Methanomicrococcus* species (95 to 97% sequence similarity) allows us to speculate cautiously that, besides H_2 and formate, methanol could also be a substrate for methanogenesis in the *Pachnoda* hindgut (54), as has already been assumed for the hindgut of *C. orthognathus* (19). Indeed, Lemke et al. (35) have shown an approximately fourfold stimulation of methanogenesis in the *Pachnoda* hindgut upon addition of methanol (5 mM) to isolated hindgut sections.

Besides methanogens, nonmethanogenic archaea affiliated with *Thermoplasmales* inhabit insect (hind)guts, as has been shown for termites (19, 53) and here for *Pachnoda* larvae. It was not possible to determine the phylogenetic relationship to clones from the wood-feeding termite *R. speratus* (53), since the overlapping region of sequences (~20 bp) was too small. As there are no cultured representatives of these mesophilic *Thermoplasmales*, the function of this group, comprising approximately 25% of the archaeal 16S rRNA genes in the *P. ephippiata* hindgut, remains to be elucidated. Clones affiliated with *Crenarchaeota* have been obtained from gut systems of fish (58), *Holothuria* (41), and soil-feeding termites (19). The clones obtained in this study were closely related to clones from the "terrestrial cluster" of *Crenarchaeota* and clustered with clones from *C. orthognathus* (97 to 98% sequence similarity to clone P1-Ar-11). Since they were the only archaeal group also occurring in the food soil, they cannot be considered as a specific gut flora, at least in *P. ephippiata* larvae.

Conclusions. The results of this study represent first and important insights into microbial community structure in the intestinal tract of humivorous beetle larvae. As with all PCR-based methods, it should be kept in mind that the relative abundance of clones for the respective community is prone to considerable bias, since several factors can affect relative rRNA gene amplicon frequencies in PCR products from mixed-template reactions (3, 57; for a review see reference 59). In a recent study with soil-feeding termites (51, 52), the results obtained with the same PCR-based methods as those used in the present study were generally in good agreement with those obtained by fluorescence in situ hybridization with group-specific probes, but there were also remarkable discrepancies, e.g., with respect to the relative abundance of *Planctomycetales*. We are presently employing fluorescence in situ hybridization to determine the absolute abundance of the members of the gut microbiota and the spatial organization of the microbial community within the *P. ephippiata* gut sections, i.e., their exact radial and axial distribution. Such information will not only serve to corroborate the results of the present study but will also help us to infer possible functions of the different phylogenetic groups detected in this study.

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3.3 Weitere Untersuchungen zur mikrobiellen Besiedlung des Darms von Rosenkäferlarven

FISH-Zählungen. Um die Gültigkeit der mit PCR-abhängigen Methoden erzielten Ergebnisse zur bakteriellen und archaellen Diversität im Verdauungstrakt von Rosenkäferlarven (Kapitel 3.2) zu verifizieren, wurde mit Zellzählungen über in-situ Hybridisierung mit fluoreszenzmarkierten, gruppenspezifischen Oligonukleotidsonden begonnen. Tabelle 3.1 fasst den momentanen Status dieser Untersuchungen zusammen. Die Ergebnisse der DAPI-Zählungen waren Gegenstand der in Kapitel 3.1 präsentierten Veröffentlichung.

Tabelle 3.1: Bisherige Ergebnisse von FISH-Zählungen, erzielt an zwei *P. ephippiata* Larven (L2), deren Darmhomogenate jeweils mit Paraformaldehyd (PFA) und Ethanol fixiert wurden. n.d.: nicht detektiert (< 0,1% aller Zellen); n.b.: nicht bestimmt. Erster Wert = Larve 1, zweiter Wert = Larve 2.

	Mitteldarm		**Enddarm**	
Zielgruppe	PFA	EtOH	PFA	EtOH
Zellen [10^{10} DAPI-Signale g^{-1} FG]	0,3 / 0,4	0,2 / 0,3	1,8 / 1,8	1,3 / 1,1
Bacteria [% DAPI]	21,9 / 19,3	49,9 / 33,9	53,8 / 50,2	67,4 / 67,5
Archaea [% *Bacteria*]	n.d.	n.d.	0,7 / 0,4	1,0 / 0,6
Actinobacteria [% *Bacteria*]	2,9 / 25,9	5,7 / 18,4	1,8 / 2,6	2,2 / 2,3
CFB-Phylum [% *Bacteria*]	0,8 / 1,5	0,2 / 0,3	9,4 / 11,9	7,1 / 6,8
Lactobacillales + *Bacillales* [% *Bacteria*]	n.b.	n.b.	16,2 [1]	18,5
TM7-Phylum [% *Bacteria*]	n.d.	n.d.	n.d.	n.d.

[1] nur Larve 2 untersucht

Mit beiden Fixierungsmitteln (insbesondere mit PFA) war der mittlere Anteil der Zellen, die ein positives Signal mit den allgemeinen *Bacteria*-Sonden gaben, im Mitteldarm deutlich geringer als im Enddarm. Dies könnte auf eine geringere Aktivität der Bakterien im Mitteldarm hindeuten. Im Mitteldarm wurden keine *Archaea*-Zellen detektiert, im Enddarm konnten dagegen Häufigkeiten von bis zu 10 *Archaea*-Zellen pro 1000 *Bacteria*-Zellen nachgewiesen werden. Diese Ergebnisse stimmen gut mit der in Kapitel 3.1 beschriebenen Beschränkung

der Methanproduktion auf den Enddarm der Rosenkäferlarven überein und bestätigen die in Kapitel 3.2 geäußerte Vermutung, dass der Mitteldarm trotz positiver *Archaea*-PCR-Amplifikate vermutlich deshalb kein Methan produziert, weil methanogene *Archaea* dort nicht abundant und/oder nicht aktiv sind.
Hybridisierungen mit der *Actinobacteria*-spezifischen Sonde konnten zumindest für eine Larve die aus Klonbibliotheken und T-RFLP-Analysen (Kapitel 3.2) ableitbare große Häufigkeit der *Actinobacteria* im Mitteldarm bestätigen. Ihre geringe Abundanz im Enddarm konnte mit beiden untersuchten Larven gezeigt werden. Zählungen mit den CFB-spezifischen Sonden bestätigten, dass Vertreter dieses Phylums im Enddarm deutlich abundanter sind als im Mitteldarm. Häufigkeiten von 26 - 44% aller Bakterien, wie sie die Klonbibliotheken und T-RFLP-Analysen nahe legten (Kapitel 3.2), konnten allerdings selbst dann nicht bestätigt werden, wenn man berücksichtigt, dass die beiden eingesetzten Sonden nur knapp 90% aller CFB-Sequenzen aus den Klonbibliotheken erfassen. Im Gegensatz dazu stimmen die Zählungen für die Gruppe der *Lactobacillales* und *Bacillales* (bislang nur Enddarm) relativ gut mit den PCR-abhängig erzielten Zahlen überein. Vertreter des TM7-Phylums stellen offensichtlich keine abundante Gruppe im Darm der Rosenkäferlarven dar, sie konnten weder mit einer spezifischen Sonde noch über T-RFLP-Analyse nachgewiesen werden.

Diversität der Pilzgemeinschaften. In Ergänzung der Untersuchungen zur bakteriellen und archaeellen Diversität im Verdauungstrakt von Rosenkäferlarven wurde auch begonnen, die Diversität der Pilzgemeinschaften in den beiden Haupt-Darmabschnitten zu charakterisieren. Hierzu wurde mit den Darmextrakten, die bereits zur Herstellung der in Kapitel 3.2 beschriebenen Klonbibliotheken von 16S rRNA-Genen benutzt wurden, eine PCR-T-RFLP-Analyse nach Lord et al. (Lord et al., 2002) durchgeführt, bei der ein ca. 650 bp langes ribosomales ITS (internal transcribed spacer) -Genfragment (gelegen zwischen dem 18S und 28S rRNA-Gen) amplifiziert wird. Die Amplifikation erfolgte nach einem Standard-PCR-Protokoll mit 35 Zyklen und 48°C Annealing-Temperatur. Als Primer wurden die bei Lord et al. (Lord et al., 2002) aufgeführten Primer EF3RCNL (FAM-markiert) und ITS4 benutzt, die Restriktionsverdaus wurden mit *Hae*III und *Taq*I durchgeführt. Die Ergebnisse zeigt Abbildung 3.1.

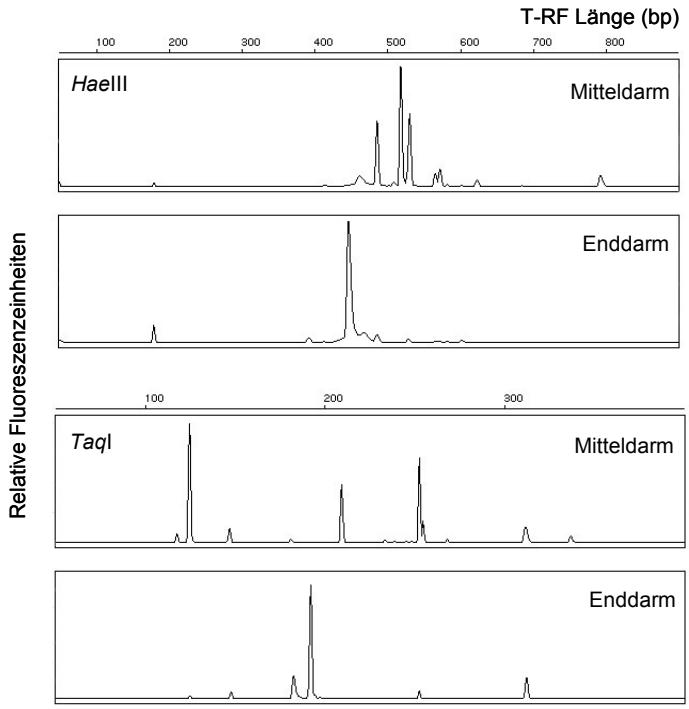

Abbildung 3.1: T-RFLP-Profile von ITS (internal transcribed spacer) -Genfragmenten zur Charakterisierung der pilzlichen Gemeinschaften in Mittel- und Enddarm von Rosenkäferlarven. Als Restriktionsenzyme wurden *Hae*III (oben) und *Taq*I (unten) benutzt.

Die ITS-Profile aus dem Darm der Rosenkäferlarven waren deutlich weniger divers als die Profile der von Lord et al. (Lord et al., 2002) untersuchten Bodenproben. Allerdings zeigten sich mit beiden verwendeten Restriktionsenzymen deutliche Unterschiede zwischen Mittel- und Enddarm. Sowohl mit *Hae*III als auch mit *Taq*I als Restriktionsenzym war die Lage der dominanten T-RFs zwischen Mittel- und Enddarm signifikant verschieden. Zu keinem der dominanten Peaks aus dem Mitteldarm gab es einen korrespondierenden dominanten Peak im Enddarm, und umgekehrt. Dies deutet auf unterschiedliche Pilzgemeinschaften in Mittel- und Enddarm der untersuchten Larven hin. Geht man von der Anzahl dominanter T-RFs aus, so scheint die Diversität an Pilzen im Mitteldarmabschnitt größer zu sein als im Enddarm, wo es (mit beiden Restriktionsenzymen) nur jeweils ein dominantes T-RF gab. Eine Amplifikation von ITS-Fragmenten aus dem Futterboden der Rosenkäferlarven war leider nicht erfolgreich, so dass die Frage der Spezifität der pilzlichen Darmflora noch ungeklärt ist. Aufgrund der deutlichen Unterschiede zwischen Mittel- und Enddarm dürfte es sich aber zumindest bei der Enddarm-Gemeinschaft nicht um eine aus dem Boden stammende Population handeln.

3.4 Physiko-chemische Gradienten und mikrobielle Diversität im Darm phytophager Maikäferlarven

Markus Egert, Ulrich Stingl, Lars Dyhrberg Bruun, Bianca Wagner, Andreas Brune und Michael W. Friedrich

Zusammenfassung: Mit dem Ziel, die Rolle intestinaler Mikroorganismen für die Ernährung ihrer Wirte und die Transformation organischer Substanz im Boden besser zu verstehen, wurden in dieser Studie physiko-chemische Bedingungen und mikrobielle Diversität in verschiedenen Darmabschnitten (Mittel- und Enddarm) und –fraktionen (Darmwand und –lumen) phytophager Maikäferlarven untersucht. Messungen mit Mikrosensoren ergaben, dass der Verdauungstrakt entlang seiner Achse leicht alkalisch (max. pH 8,6) ist und zum Rectum hin neutral wird. Mittel- und Enddarm waren überwiegend anoxisch, Sauerstoff drang max. 100 µm tief in die Darmperipherie ein. Während das Redoxpotential im Mitteldarm stark positiv war, sank es im Enddarm auf bis zu 0 mV ab. Methan wurde nur vom Enddarm gebildet; dies stimmte gut mit dem Befund überein, dass keine archaeellen 16S rRNA-Gene aus dem Mitteldarm amplifiziert werden konnten. Acetat war in beiden Darmabschnitten bei weitem die häufigste kurzkettige Fettsäure (15 - 34 mM), der Mitteldarm enthielt auch relativ hohe Konzentrationen an Glucose (12 mM), die vermutlich aus der Hydrolyse pflanzlicher Polysaccharide stammt. Mittel- und Enddarm waren dicht mit Mikroorganismen besiedelt. T-RFLP-Analyse mikrobieller 16S rRNA-Gene zeigte, dass sich im Mitteldarm keine stabile Mikroflora befindet, wohingegen die bakterielle Gemeinschaft an der Enddarmwand eine sehr hohe Ähnlichkeit zwischen verschiedenen Larvenindividuen aufwies. Diese Ergebnisse sprechen gegen eine spezifische, symbiontische Bedeutung von Mikroorganismen im Mitteldarm, deuten aber gleichzeitig eine solche für die Bakterien an der Enddarmwand an. Relativ hohe Sulfatkonzentrationen in Mittel- und Enddarm (0,9 - 2,9 mM) stimmten gut mit dem Vorkommen Sulfat reduzierender Bakterien an der Enddarmwand überein, worüber in der Begleitstudie (Kapitel 3.5) im Detail berichtet wird.

Physicochemical gradients and microbial diversity in the intestinal tract of the phytophagous larva of *Melolontha melolontha* (Coleoptera: Scarabaeidae)

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Abstract

Scarabaeid beetle larvae play an important role in the transformation and mineralization of organic matter in soil. During gut passage, organic matter is exposed to the physicochemical conditions and the activity of a diverse gut microbiota in different gut compartments. In this study we investigated the physicochemical conditions and the microbial diversity in the two major gut compartments (midgut and hindgut) and subcompartmental gut fractions (gut wall and lumen) of the phytophagous larva of *Melolontha melolontha*, an important plant pest in Central Europe. Microsensor measurements revealed slightly alkaline intestinal pH values (max. pH 8.6) along the gut axis, reaching more neutral values towards the rectum. Midgut and hindgut were largely anoxic, and oxygen did not penetrate deeper than 100 µm into the gut lumen. The midgut displayed positive redox potentials, which dropped to 0 mV in the large hindgut dilatation. Methane emission was restricted to the hindgut compartment, which was in agreement with the failure to PCR-amplify archaeal 16S rRNA genes from midgut DNA extracts. Acetate was the by-far most abundant (15 - 34 mM) short chain fatty acid in both gut sections. The midgut contained considerable amounts of glucose (12 mM), probably originating from the hydrolysis of plant polysaccharides. Both gut compartments, but particularly the hindgut, were densely populated with microorganisms. T-RFLP analysis of microbial 16S rRNA genes indicated that the midgut compartment lacks a specific and stable microbiota, whereas the bacterial community at the hindgut wall was very similar among individual larvae. High sulfate concentrations (0.9 - 2.9 mM) in both gut sections were in agreement with the occurrence of sulfate-reducing bacteria in the hindgut wall community of the *Melolontha* larva, which was in detail analyzed in a companion study (M. Egert, L. Dyhrberg

Bruun, B. Wagner, A. Brune, and M.W. Friedrich, Appl. Environ. Microbiol. X: XXXX – XXXX, 200X). Our results indicate that microorganisms in the midgut, which lacks a stable community, might not fulfill a particular symbiotic function, whereas the bacterial community at the hindgut wall, characterized by a high similarity among individual larvae, probably does.

Introduction

The *Scarabaeidae* are a large beetle family comprising ca. 25.000 different species worldwide (35). The larvae of scarabaeid beetles ("white grubs") comprise species from detrivorous, humivorous, coprophagous and phytophagous feeding guilds and are, as soil macroinvertebrates in general, considered to be important for the transformation of organic matter in soil (37,61).

Steep axial and radial gradients of the physicochemical conditions are characteristic features of insect intestinal systems (7), thought to have important functions for digestion. In soil feeding termites, for instance, the extreme alkalinity of the anterior hindgut and the influx of oxygen lead to a decrease in molecular weight, an increase in solubility and to chemical oxidation of the ingested soil organic matter, which render it more accessible for digestion in subsequent less alkaline gut compartments (29-31). In other insects, high intestinal pH-values are interpreted as an adaptation to a diet rich in tannins or phenols, preventing a precipitation of dietary proteins and digestive enzyme (20,42). The physicochemical gradients in different gut compartments are known as a shaping force for the microbial communities present there. The influx of oxygen into the termite gut allows the occurrence of aerobic microorganisms (9), the fermentation balance of which is strongly influenced by oxygen (54). Therefore, investigations about microbial communities in insect guts should be linked to the physicochemical conditions in the investigated compartments to gain a deeper understanding of the ongoing digestive processes.

The intestinal tract of scarabaeid beetle larvae comprises two major sections, a tubular, alkaline midgut, equipped with hydrolytic enzyme activities, and a bulbous, circumneutral hindgut, considered to function as a fermentation chamber (22,48,58,59). Both compartments contain a dense microbiota (1,10), which was early recognized to participate in digestion (56,57). However, little is known about its exact composition or distribution within the gut. Detailed studies about digestive processes are restricted to a few species (*Costelytra zealandica*, (1,2);

Oryctes nasicornis, (3,4); *Pachnoda marginata* (11)). Recently we reported about physicochemical conditions, microbial activities and microbial community composition in the intestinal tract of humivorous scarabaeid beetle larvae (*Pachnoda ephippiata*, *Cetoniinae*) (19,39). Comparable to soil-feeding termites, the *Pachnoda* gut was characterized by strong midgut alkalinity, high concentrations of microbial fermentation products and a dense and diverse microbial community which differed clearly between the major gut sections and was clearly different to the community in the ingested soil. However, little is know about scarabeid larvae from other feeding guilds.

In order to gain more insight into the digestive processes and microbial associations of phytophagous scarabaeid beetle larvae, we investigated the physicochemical conditions and the microbial diversity in different compartments of the intestinal tract of *Melolontha melolontha* larvae. Cockchafers of the genus *Melolontha* ("maybeetles"; *Melolonthinae*) belong to the insects most public in Central Europe, where they have been know as severe pests of agricultural plants for more than 500 years (33). The larvae of *M. melolontha* develop in the soil of open fields for 3 - 4 years, depending on the local climate (34). They are phytophagous, feeding on plant roots, which can lead to severe plant damage, e.g. in grass lands, wine yards or orchards.

Molecular fingerprinting of 16S rRNA genes revealed that the hindgut wall of *M. melolontha* larvae contains a diverse bacterial community which was, however, very similar among individual larvae. On the contrary, the bacterial community in the midgut compartment differed greatly among the investigated larvae, indicating a more transient intestinal flora in this compartments. To elucidate the composition and topology of the microbial community in the hindgut of *M. melolontha* larvae in detail, we performed a 16S rRNA gene cloning approach accompanied by FISH analyses, the results of which are reported in a companion paper (17).

Materials and Methods

Sampling and dissection of *Melolontha melolontha* larvae. Larvae (late second and third instars) of the European cockchafer (*Melolontha melolontha* L.) were collected in 2002 and 2003 near Obergrombach, Germany. The insects were transported to Marburg and incubated separately in sealed plastic beakers at 15°C for several months. The larvae were kept in soil and fed with grass roots obtained from a meadow close to the institute, respectively. Water

was added *ad libitum*. Only feeding and healthy insects were used for the experiments. For dissection, larvae were anaesthetized by exposure to a N_2/CO_2 (80:20, v/v) gas atmosphere for about 20 min. Subsequently, the larvae were transferred to ice-cold Ringer`s solution (8) and decapitated. The whole gut was excavated and the fat cells were removed. For separation of different gut sections and compartments, isolated guts were separated at the muscular midgut-hindgut junction into a midgut section, including the short foregut, and a hindgut section, including paunch, colon and rectum; no ligation was necessary to prevent leaking of the gut contents. For preparation of wall and lumen fractions isolated gut sections were carefully opened with scissors and gut content was taken out with a sterile spatula. Subsequently, gut walls were repeatedly washed with sterile Ringer`s solution (8) until total removal of attached gut content.

Microsensor measurements. For the measurements, freshly dissected guts were embedded in a small PVC chamber (8 x 1.3 x 1.3 cm) and tightened onto the silicon covered bottom with minute steel pins. The chamber was constantly flushed with air-saturated insect Ringer` solution (8) by means of a peristaltic pump (5 ml min^{-1}). Microsensors were positioned with a manual micromanipulator and progress of the tip was monitored with a horizontally mounted stereomicroscope. All measurements were carried out at ambient temperatures (22 ± 1°C). A more detailed description of the setup has been published elsewhere (12).

Oxygen measurements were performed using Clark-type sensors with guard cathodes (45), which were constructed and calibrated as described previously (8). Polarographic hydrogen microsensors had the same principle design (60), but the working electrode was covered with platinum black. (15). Testing and calibration procedures were performed as described previously (15). Both microsensors had a 90% response times of < 5 s and tip diameters of 10 - 15 µm. The stirring sensitivity of both electrodes was < 1% of the signal obtained upon calibration in air-saturated water or at 20 kPa H_2, respectively.

For pH measurements, capillary microsensors with ion-selective membranes (LIX-type) with a 200 - 300 µm pore size membrane of Hydrogen ionophore I - cocktail A (Fluka, Buchs, Switzerland) were constructed using the design described in (46). The microsensors were equipped with an external casing filled with 1 M KCl to minimize electrical noise (28) and had tip diameters of 10 - 30 µm and 90% response times of < 10 s. The calibration was performed in commercial pH standard solutions (pH 5 - 12) and showed a log-linear response in this range. Electrically shielded platinum redox microelectrodes with response times of 10 -

20 s and tip diameters of 10 - 20 µm were constructed and calibrated with freshly prepared quinhydrone solutions as described elsewhere (15). In both cases, the working electrode was connected to a high-impedance electrometer amplifier ($R_i > 10^{14}$ Ω) via a low-noise coaxial cable, and electrode potentials were measured against a Ag/AgCl-electrode which was in direct contact with the measurement microchamber via a KCl-filled agar bridge (1% Agar in 1 M KCl). Reference and casing were grounded.

Metabolites in gut fluids and hemolymph. Gut sections were dissected as described above. Hemolymph was collected by cutting the dermis before decapitating the larvae. Both were treated as follows: mild sonification, centrifugation (14000 *g*, 10 min), addition of 5 µl H_2SO_4 (5 M) per 95 µl supernatant, centrifugation (14000 *g*, 10 min), HPLC measurement of supernatant (50 µl). Acetate, glucose, butyrate, propionate, succinate, formate, lactate, ethanol, iso-butyrate and iso-valerate served as external standards (2.5 mM, 5 mM and 10 mM, each). The HPLC system was equipped with a Resin ZH 8 µm column (250 x 8 mm, Grom, Herrenberg, Germany) and a precolumn containing the same material (Sugar 3, Grom, Herrenberg, Germany). Analyses were performed at 60°C and 25 bar with H_2SO_4 (5 mM) as eluent (flow rate 0.6 ml min^{-1}). The eluent was connected to an online degasser (DGU-14A, Shimadzu, Duisburg, Germany) to prevent air bubbles in the system. Detection of metabolites was performed using a RI detector (RID-10A, Shimadzu, Duisburg, Germany). For additional identification and reassignment of peaks to standard compounds an additional in line UV detector (SPD-10A, λ = 210 nm, Shimadzu, Duisburg Germany) was used. The detection limits were as follows: acetate (0.23 mM), glucose (0.04 mM), butyrate (0.16 mM), propionate (0.15 mM), succinate (0.06 mM), formate (0.31 mM), lactate (0.11 mM), ethanol (0.39 mM), iso-butyrate (0.10 mM), iso-valerate (0.11 mM). Data recording and integration were performed with the Eurochrom 2000 software package (Knauer, Berlin, Germany).

Sulfate concentration. For determination of the sulfate concentration isolated midguts and hindguts (n = 4, each) were homogenized with an ultrasonic probe (Dr. Hielscher, Teltow, Germany) and centrifuged for 15 min at 21.000 *g*. The supernatants (~ 50 µl) were 1:1 diluted with HCl (0.5 M) and again centrifuged (10 min, 10.000 *g*). Subsequently, sulfate concentration in the supernatants were determined by ion chromatography analysis using a Sykam LCA-A03 column (Sykam, Fürstenfeldbruck, Germany) at 30°C with an aqueous eluent containing Na_2CO_3 (5 mM), 4´-hydroxybenzonitrile (50 mg l^{-1}) and 20% acetonitrile at a flow rate of 1.5 ml min^{-1}. The column was regenerated with 0.2 M H_2SO_4. The presence of sulfate

was ensured by analyzing each sample twice, i.e. before and after addition of extra sulfate, which always increased the peak supposed to represent sulfate.

Methane production rates. Larvae or isolated gut sections were placed into 150 ml or 36.5 ml glass vials, respectively, which were sealed (under air or N_2-atmosphere, respectively) with rubber stoppers, and were incubated for several hours at room temperature in the dark. If individual gut compartments were tested, the vials received a small amount of insect Ringer`s solution (8) to avoid desiccation of the samples; care was taken not to cover the segments with liquid in order to facilitate the gas exchange with the headspace. At regular intervals, gas samples were taken and analyzed for methane by gas chromatography using a flame ionization detector (Shimadzu, GC 8 A, Japan). Stimulation of methane emission was tested by supplementing the headspace with H_2 (5%) (larvae and gut sections) or by adding Na-formate (5 mM; gut compartments only), after the basal rate of methane production had been established.

Total cell counts. Cell numbers were obtained following a standard protocol (44) with the following modifications: gut sections were homogenized with sterile glass homogenizers, appropriately diluted with phosphate-buffered saline (PBS) solution and fixed with paraformaldehyde (3%, w/v) overnight at 4°C in the dark. Fixed homogenate (1 ml) was filtered onto white polycarbonate membrane filters, washed with PBS, air-dried, and subsequently stained with 4,6-diamino-2-phenlyindol (DAPI). Cells were counted by epifluorescence microscopy with an ocular grid; approximately 1000 – 2000 cells were counted for each sample.

DNA extraction. DNA was extracted from different gut compartments and fractions (midgut lumen, midgut wall, hindgut lumen, hindgut wall) of individual larvae and from plants roots with some attached soil, thought to represent food consumed by the larvae, (max. 0.5 g) following a direct lysis protocol that has been previously described in detail (25). DNA was purified from the supernatant with phenol/chloroform/isoamylalcohol (25:24:1), followed by consecutive isopropanol and ethanol precipitation. Subsequently, DNA extracts were cleaned with polyvinylpolypyrrolidone-filled spin-columns as described previously (19).

T-RFLP analysis. 16S rRNA genes were specifically amplified using the primer combination of 6-carboxyfluorescein (FAM)-labeled primer 27f (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) (16) and 907r (5′-CCG-TCA-ATT-CCT-TTR-AGT-TT-3′) (43) for *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) (23) and FAM-labeled Ar915r (5′-GTG-CTC-CCC-CGC-CAA-TTC-CT-3′) (41) for *Archaea*. The standard reaction mixture contained, in a total volume of 50 µl, 1x PCR buffer II (Applied Biosystems, Weiterstadt, Ger-

many), 1.5 mM $MgCl_2$, 50 µM of each of the four deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 µM of each primer (MWG Biotech, Ebersberg, Germany), and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). In addition, 1 µl of a 1:20 dilution of DNA extract was added as template. All reactions were prepared at 4°C in 0.2 ml reaction tubes to avoid non-specific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94°C) block of a Gene Amp 9700 Thermocycler (Applied Biosystems). The standard thermal profile for the amplification of 16S rRNA genes was as follows: initial denaturation (94°C, 3 min), followed by 32 (*Bacteria*) or 35 (*Archaea*) cycles of denaturation (94°C, 30 s), annealing (52°C, 45 s) and extension (72°C, 90 s). After terminal extension (72°C, 5 min), samples were stored at 4°C until further analysis. Aliquots (5 µl) of 16S rRNA gene amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide. PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany).

Prior to digestion, amplicon concentrations were determined photometrically. 75 ng of DNA, 2.5 U of restriction enzyme (*Msp*I [bacterial amplicons] or *Alu*I [archaeal amplicons]; Promega, Mannheim, Germany), 1 µl of 10x incubation buffer, and 1 µg of bovine serum albumin were combined in a total volume of 10 µl and digested for 3 h at 37°C. Fluorescently labeled T-RFs were size separated on an ABI 373A automated sequencer (Applied Biosystems) using an internal size standard (GeneScan-1000 ROX; Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems).

Ecological indices and statistical analysis. Prior to statistical analysis, T-RFLP electropherograms (starting from 50 bp to exclude T-RFs caused by primer dimers) were normalized to identical total peak heights, respectively, using an iterative method described by Dunbar et al. (14), where all T-RFs with a height less than 50 relative fluorescence units were omitted. All subsequent calculations of ecological indices were performed with major T-RFs, arbitrarily defined as those with a relative peak height of ≥ 1.5% of the total electropherogram peak height. To ensure that the relative heights of all major T-RFs equal 100%, they were normalized to the total peak height of all major T-RFs per electropherogram. As the size of identical T-RFs can vary in a range of 1-2 bp among different gels and/or lanes of the same gel, major T-RFs similar in size of ± 1-2 bp were summarized to operational taxonomic units (OTUs).

For the calculation of ecological indices, OTUs were treated as species and their relative height served as a measure of relative abundance. Shannon-Wiener indices and (Shannon-)

evenness (26) were used to compare diversity among different samples. Evenness values were calculated using the natural logarithm of the number of OTUs per single electropherogram. To describe the pair wise similarity of microbial communities, Morisita indices of community similarity were used as described in (13). Morisita indices range from 0 to 1, with 1 indicating complete (100%) identity of two communities. Differences between samples (in ecological indices and physicochemical parameters) were checked for statistical significance with non-parametric tests (Mann-Whitney) using SYSTAT 10.0 (SPSS Inc., Chicago, USA).

Results

Cell densities. The intestinal tract of *M. melolontha* larvae (Fig. 1) comprises two major compartments – a long, tubular shaped midgut and a more spheroidal hindgut (Fig. 1). Both gut compartments are colonized by prokaryotic microorganisms, with cell densities of 3.2 (± 1.2) x 10^9 cells g^{-1} FW in the midgut and 1.5 (± 0.6) x 10^{10} cells g^{-1} FW in the hindgut (DAPI-counts; n = 5, each). On an average, the hindgut compartments contained approximately 5-fold more cells than the midguts, and this difference was significant (P = 0.014).

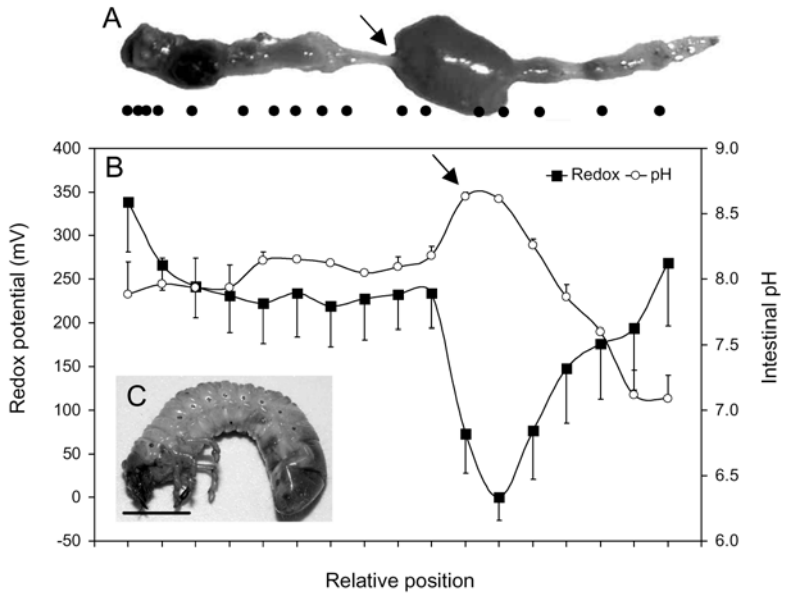

Figure 1. A). Excised gut of a *Melolontha melolontha* larva (late second larval instar; ca. 3.5 cm long). Measurement points for axial gut profiles of physicochemical parameters are indicated by dots. B). Profiles of redox potential and intestinal pH along the gut axis of *M. melolontha* larvae (late L2). Each value represents mean ± SEM of three replicate guts. The arrow indicates the junction of midgut and hindgut. C) Late second larval instar (L2) of *M. melolontha*. Scale bar represents approximately 1 cm.

Microsensor measurements. Microsensor measurements performed with intact intestinal tracts incubated in aerated Ringer's solution revealed that the guts of the investigated *Melolontha* larvae (n = 3) were characterized by moderate pH and large redox potential dynamics (Fig. 1). The intestinal pH along the midgut region was quite stable, ranging from 7.9 to 8.2 (± 0.0 - 0.2). The highest pH-values were recorded in the anterior hindgut (pH 8.6 ± 0.0), from where they constantly declined to neutral values (pH 7.1 ± 0.2) at the end of the rectum. Redox potential (E_h) was in the range of 220 - 340 ± 30 - 60 mV (Fig. 1), which indicated that the midgut content of *Melolontha* larvae is characterized by oxidizing conditions. At the junction of midgut and hindgut, E_h-values sharply dropped to reach the lowest values (0 ± 30 mV) at the anterior hindgut. Towards the end of the hindgut and along the rectum E_h-values steadily increased again to reach values comparable to those in the midgut (270 ± 70 mV).

Radial oxygen concentration profiles revealed steep gradients around the mid- and hindgut compartments; in no case (n = 2) did O_2 penetrate deeper than 100 µm into the lumen of the respective segments. The complete consumption of O_2 in the gut periphery led to anoxic conditions at the gut center of midgut and hindgut (details not shown). Axial hydrogen concentration profiles (n = 4) showed that the accumulation of hydrogen (0.1 - 0.4 ± 0.0 - 0.2 kPa) was restricted to a small region of the midgut compartment (measurement points 4 - 7 in Fig. 1; details not shown).

Methane production rates. The methane emission rate of individual larvae (n = 5), incubated under air, was 51 ± 9 nmol g^{-1} FW h^{-1}. Addition of 5% hydrogen to the headspace significantly (P = 0.016) doubled the emission rate to 96 ± 7 nmol g^{-1} FW h^{-1}. Separate incubation of isolated midguts and hindguts (n = 3, each; under N_2-atmosphere) revealed that CH_4 emission was restricted to the hindgut compartments. They produced methane at a rate of 23 ± 10 nmol g^{-1} FW h^{-1}, which corresponded to approximately half of the rate observed with living animals. When incubated with air, isolated hindguts revealed emission rate comparable to those obtained under N_2-atmosphere (22 ± 13 nmol g^{-1} FW h^{-1}). Addition of 5% hydrogen increased the average methane emission to 44 ± 17 nmol g^{-1} FW h^{-1}. However, due to large individual variations, this increase was not significant. Subsequent addition of formate (5 mM), another potential methanogenic substrate, did not affect methane emission from isolated hindguts (26 ± 12 nmol g^{-1} FW h^{-1}).

Microbial fermentation products and sulfate. Both compartments of the intestinal tract of *Melolontha* larvae contained short-chain fatty acids typical for microbial fermentations, with

acetate being by far the dominant one (Table 1). The concentrations of acetate and succinate were significantly (P = 0.042 and 0.020, respectively) higher in the midgut than in the hindgut, while lactate and propionate concentrations did not differ significantly among the two compartments. The spectrum of fatty acids in the hemolymph was similar to that in the gut sections, however, the acetate concentration was significantly lower than in midgut (P = 0.021) and hindgut (P = 0.020). The concentration of glucose was by far highest in the midgut (P = 0.021), while it was not different between hindgut and hemoloymph.

Table 1. Concentration of short-chain fatty acids and glucose (mM) in midgut, hindgut, and hemolpyph of *Melolontha melolontha* larvae. Values are mean ± SEM (n = 4). n.d. = not detected. Means within rows followed by the same letter are not significantly (P < 0.05) different.

	Midgut	Hindgut	Hemolymph
Acetate	34 ± 6 a	15 ± 3 b	3.2 ± 1.2 c
Lactate	1.8 ± 0.1 a	0.4 ± 0.2 a	1.4 ± 0.5 a
Propionate	n.d. [1] a	0.2 ± 0.2 a	n.d. [1] a
Succinate	0.8 ± 0.2 a	0.2 ± 0.1 b	2.9 ± 0.9 a
Glucose	12 ± 4 a	0.9 ± 0.4 b	2.9 ± 0.9 b

[1] detection limit: 0.15 mM propionate

The concentration of sulfate was determined to be 2.9 ± 0.6 mM (n = 5) in the midgut, which was significantly (P = 0.014) higher than in the hindgut (0.9 ± 0.3 mM; n = 5).

Microbial diversity in different gut compartments and fractions. The bacterial diversity in different gut compartments (midgut and hindgut) and subcompartmental fractions (lumen and gut wall) of three individual *M. melolontha* larvae and of two root/soil samples, thought to represent some of the larval food, was analyzed with T-RFLP fingerprinting of 16S rRNA genes (Fig. 2). While the profiles obtained from the hindgut fractions consistently displayed a high diversity of T-RFs, the majority of midgut profiles were of very low diversity, i.e. only 1- 3 T-RFs were present. In two profiles just one T-RF (495 bp) was present, implying that possibly only one species was present. This 495 bp T-RF was also present in two other profiles (midgut lumen and wall 3; Fig. 2), where it was associated with other T-RFs. However, it

was absent in two other profiles (midgut lumen and wall 2; Fig. 2), which also showed a much higher diversity than the other midgut profiles. The very low diversity in the majority of midgut samples was quite surprising, since (as expected) the bacterial diversity on the roots (and in the soil attached to them), consumed by the larvae, is quite high, as demonstrated by two fingerprints depicted in Fig. 2. Because the midgut profiles varied greatly with respect to T-RF diversity and obviously lacked consistency, we did not calculate ecological indices for this compartment.

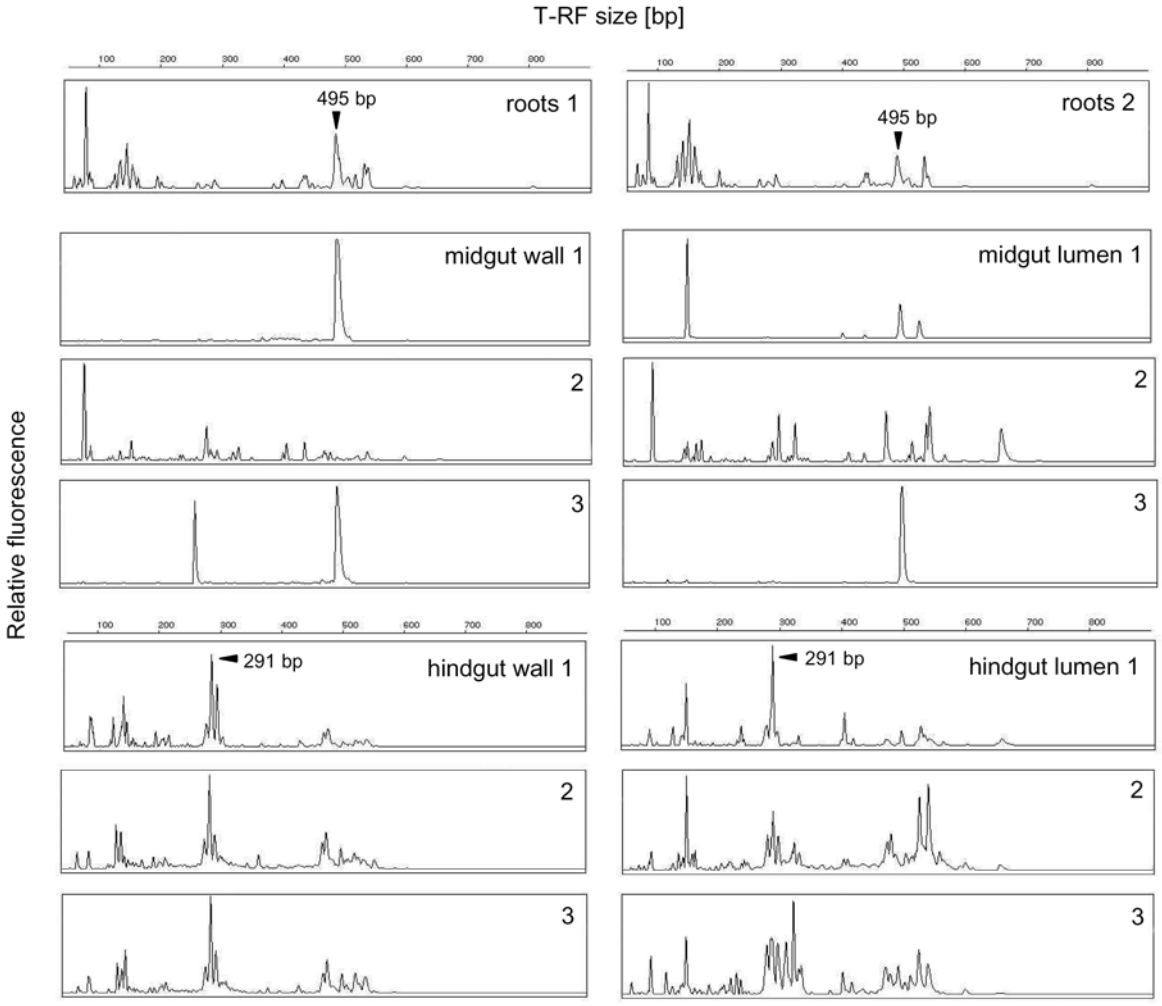

Figure 2. Bacterial 16S rRNA gene T-RFLP-profiles obtained from DNA extracts of three individual *M. melolontha* larvae (#1 - 3) and from two roots samples with attached soil, representing potential food of the larvae. The excised guts were separated into compartments (midguts and hindguts) and each compartment into two subcompartmental fractions (gut wall and lumen). Prominent peaks referred to in the text are indicated. *Msp*I was used for restriction digest.

However, the three hindgut profiles obtained from the lumen and wall fractions, respectively, displayed similarities, in particular all hindgut wall profiles were characterized by a prominent 291 bp T-RF.

Ecological indices, calculated from the T-RFLP profiles, are given in Table 2. Based on Shannon-Wiener indices and evenness values, bacterial OTU diversity of the hindgut wall and lumen fractions of *M. melolontha* larvae was not significantly different. Morisita indices of community similarity revealed, that the three investigated hindgut wall profiles posses an average similarity of almost 90%, which was significantly ($P = 0.046$) greater than the average similarity of the hindgut lumen profiles (68%).

Table 2. Diversity and similarity indices, based on 16S rRNA gene T-RFLP profiles, characterizing the bacterial community at the hindgut wall and in the hindgut lumen of *Melolontha melolontha* larvae. Values are mean ± SEM (n = 3). Means within rows followed by the same letter are not significantly ($P \leq 0.05$) different.

Index	hindgut wall	hindgut lumen	
Diversity (Shannon-Wiener)	2.78 ± 0.02 a	2.88 ± 0.10 a	
Evenness	0.93 ± 0.01 a	0.93 ± 0.02 a	
	wall : wall	lumen : lumen	wall : lumen
Similarity (Morisita)	0.89 ± 0.03 a	0.68 ± 0.01 b	0.72 ± 0.05 b

Moreover, the average similarity among the three hindgut profiles, obtained from three individual larvae, was significantly ($P = 0.050$) higher than the average similarity between corresponding wall and lumen profiles, i.e. profiles belonging to the same larva, which was 72%.

The archaeal diversity in the intestine of *M. melolontha* larvae was also assessed by T-RFLP analysis. However, we never obtained an archaeal PCR-signal from any *Melolontha* midgut sample (data not shown). In case of the hindgut, T-RFLP profiles of total hindguts (n = 3) as well as wall and lumen fractions (Fig. 3) uniformly displayed a dominant 67 bp and a smaller 167 bp T-RF, which was identified as a pseudo-T-RF (18) (data not shown).

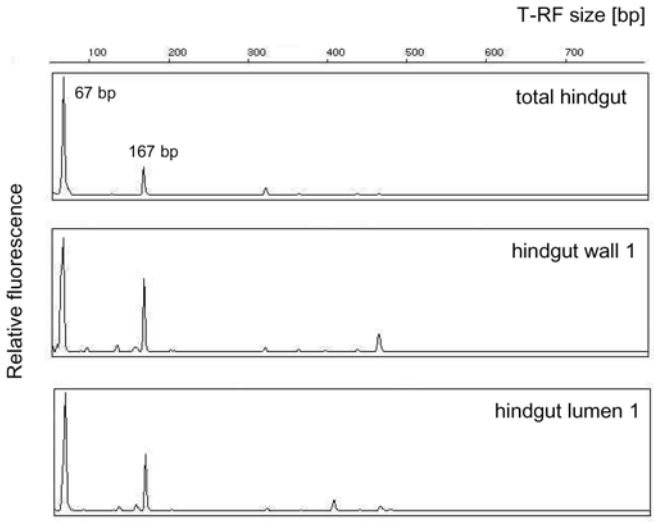

Figure 3. Archaeal 16S rRNA gene T-RFLP profiles of DNA extracts obtained from a total hindgut of a *M. melolontha* larvae, and from another hindgut separated into gut wall and lumen fraction.

Discussion

This study represent the first comprehensive report on physicochemical gradients and the bacterial and archaeal diversity in the two major gut sections (midgut and hindgut) of a phytophagous beetle larva (*Melolontha melolontha; Scarabaeidae).* Our results show, that the physicochemistry of the *Melolontha* gut is characterized by pronounced axial (e.g. intestinal pH, redox-potential, concentration of metabolites) and radial (oxygen) gradients, which is paralleled by distinct differences in the colonization with microorganisms. The finding that the bacterial community at the hindgut wall of *Melolontha* larvae is highly similar among individual larvae encouraged us to investigate the microbial diversity in the hindgut section of *Melolontha* larvae in more detail, which is reported in the companion study (17).

Physicochemical gut conditions. The intestinal pH-values determined in this study corroborate values reported by Wildbolz already in 1954 (59). Compared to soil- or litter-feeding scarabaeid larvae, pH-values along the whole midgut of phytophagous *M. melolontha* larvae were just slightly alkaline (~ pH 8.0). For example, maximum pH values determined from the midgut of humivorous larvae of *Osmoderma scabra* and *Cotnis nitida* (22), *Oryctes nasicornis* (3) and *Pachnoda ephippiata* (38) were between pH 10 - 12. High intestinal pH-values are interpreted as special adaptations to the preferably consumed food. Soil-feeding termites perform an alkaline extraction of the ingested soil organic matter, which facilitates the desorption of organic matter from the soil matrix and decreases its molecular weight (31). In phyto-

phagous lepidopteran and detrivorous dipteran larvae high intestinal pH-values prevent precipitation of digestive enzymes and/or dietary proteins caused by tannins and/or phenols enriched in their diet (20,42). Assuming a diet consisting of plant root material, probably not particularly rich in phenols or tannins and not as hard to extract as soil organic matter, there is apparently no need for an extreme alkaline pH in the *Melolontha* midgut. However, the midgut of phytophagous *Costelytra zealandica* (*Melolonthinae*) larvae showed maximum pH-values of almost pH 11.0 (5). Maybe these different pH-values reflect different dietary preferences. For *Costelytra zealandica* the high pH-values were interpreted as an adaptation to rapidly extract alkali-soluble polysaccharides from the cell walls of white clover root cells, which are the preferred food of these larvae and substantially degraded during the gut passage (2,5).
The pH-values determined for the large *Melolontha* hindgut were with pH 7.9 - 8.6 comparable to those obtained for other scarabaeid larvae, e.g. *Oryctes nasicornis* (pH 8.5, (3)) or *Costelytra zealandica* (pH 8.2, (5)).
Both mid- and hindgut of the investigated larvae were anoxic in their center and at the gut periphery oxygen was penetrating into the gut lumen not deeper than 100 µm. These results are comparable to those obtained with (L2 and L3 instars of) *Pachnoda ephippiata* larvae (38). Anoxia is possibly contributable to microbial respiration and chemical oxygen consumption of the alkaline gut content, as also shown for soil-feeding termites (31). In contrast to the L2 and L3 instars of *Pachnoda ephippiata* (ca. –175 to 75 mV; (38)) or the larva of *Oryctes nasicornis* (30 mV; (3)) redox potentials along the whole midgut of *Melolontha* larvae were noticeably positive. E_h values in the hindgut paunch, though much lower than in the midgut, were also higher than those obtained for *Pachnoda ephippiata* (ca. –25 to –125 mV) or *Oryctes nasicornis* (–80 to –100 mV; (3)). Higher redox potentials in the midgut of the phytophagous *Melolontha* larvae might be attributable to the lower (compared to soil-feeding scarab larvae) pH-values there. In case of the hindgut a lack of electron acceptors, such as humic substances and/or iron minerals, which comprise a considerable part of the diet of soil-feeding insects (32), might be another explanation.
Short chain fatty acid and sulfate concentrations. Hydrolysis of polymers in the intestinal tract of *Melolontha* larvae is most likely performed by both host and microbial enzymes, which can, however, here not be differentiated in detail. Depolymerizing enzymes have been found in the intestinal tract of different scarabaeid beetle larvae. *Cetonia aurata* and *Potosia cuprea* possess alkaline protease and amylase activities in the midgut and cellulase activity in

the hindgut (49,56). For *Oryctes nasicornis*, cellulose hydrolysis was proven for mid- and hindgut (4) and also *Pachnoda marginata* larvae possess high activities of hydrolytic enzymes (xylanase, carboxymethylcellulase) (11) in both mid- and hindgut. A complex mixture of proteinases was recently described for the midgut of *Melolontha melolontha* larvae (55), high protease activity also for the midgut of *Costelytra zealandica* (5). In addition, phytophagous *Costelytra zealandica* larvae also displayed amylase and invertase activity in the midgut and xylanse activity in the hindgut. From the absence of any cellulase activity it was suggested, that these larvae, belonging to the same family as *M. melolontha*, do not utilize structural plant polysaccharides such as cellulose (1). However, for the hindgut of *M. melolontha*, carboxymethylcellulase activity was reported (47), and as already pointed out in (4), the absence of any measurable cellulase activity does not exclude celluose hydrolysis, since bacterial cellulases remain bound to the bacterial surface and are difficult to detect.

The spectrum of different fermentation products found in the midgut and hindgut fluids suggests that also the gut microorganisms participate in the fermentative breakdown of the products of enzymatic hydrolysis. Acetate was by far the dominant short-chain fatty acid, particularly in the midgut (> 30 mM). In *Oryctes nasicornis* (3,4) and *Pachnoda* species (10,11,38) acetate was also among the dominant fatty acids, however, absolute concentrations were considerably lower (e.g. 4 – 8 mM in the midgut of L2 and L3 *Pachnoda ephippiata* larvae; (38)) and other fatty acids (lactate and propionate, respectively) reached comparable concentrations. However, acetate was the dominant short chain fatty acid in *Costelytra zealandica* larvae and also reached concentrations comparable to those reported here (ca. 25 mM in midgut and hindgut; (1)). The high glucose concentration in the midgut most likely stems from the hydrolytic breakdown of plant polysaccharides, the significant lower concentration in the hindgut is probably due to microbial fermentations, which is corroborated by the high diversity of bacterial clones related to hydrolytic, cellulolytic, and fermentative microorganisms (*Clostridiales*, *Bacteroidetes*), reported in the companion study (17).

The spectrum of short chain fatty acids in the hemolpyph was quite similar to the spectrum in the intestinal tract, indicating that the microbial fermentation products, and also the glucose, are resorbed and eventually oxidized by the insect (6). Short chain fatty acids in the hemolymph have also been reported for the larva of other scarabaeid beetles, e.g. *Oryctes nasicornis* (3) and *Pachnoda ephippiata* (38).

The determined sulfate concentrations were quite high, particularly in the midgut compartment (2.89 mM). Sulfate concentrations in the hindgut were with 0.93 mM resembling values determined for wood-feeding termites and a wood-feeding roach (0.3 – 0.7 mM; (36)). The occurrence of sulfate in midgut and hindgut suggests an uptake of sulfate with the food (plant root material, soil particles). However, the sulfate concentration in the food (the exact composition of which is unknown) was not determined yet, so that it is unclear if the sulfate concentration in the ingested food is sufficient to explain the intestinal sulfate concentrations. The significant decrease of the sulfate concentration from midgut to hindgut suggests a consumption of sulfate in the hindgut, which is corroborated by the occurrence of sulfate reducing bacteria in high numbers at the hindgut wall of *Melolontha* larvae, reported in the companion study (17).

Methanogenesis. In *M. melolontha* larvae methanogenesis was restricted to the hindgut. This finding is in good agreement with the fact that no archaeal PCR-products could be obtained from any *Melolontha* midgut sample, and with the occurrence of clones related to *Methanobrevibacter*-species in the hindgut of *Melolontha* larvae, reported in the companion study (17). Also in other scarabaeid larvae methanogenesis occurred exclusively in the hindgut (3,24,38). The emission rates of individual larvae were with ca. 50 nmol CH_4 g^{-1} FW h^{-1} lower than those obtained for humivorous *P. ephippiata* larvae (120 – 360 nmol CH_4 g^{-1} FW h^{-1}; (38)) and other cetoniid species (255 nmol CH_4 g^{-1} FW h^{-1}; (24)), however, they were comparable to those obtained for *Oryctes nasicornis* larvae (ca. 40 nmol CH_4 g^{-1} FW h^{-1}; (3)). Interestingly, methane emission could not be shown for *Costelytra zealandica*, belonging to the same family (*Melolonthinae*) as *M. melolontha*. (1). Isolated hindguts displayed a ~50% lower methane emission rate than total individuals, an effect that was even more pronounced observed with cockroaches, soil-feeding termites and *Pachnoda ephippiata* larvae (38,39,50). While in cockroaches and soil-feeding termites hydrogen from adjacent gut regions is thought to drive methanogenesis in the hindgut (39,50), a formate shuttle from the midgut via the hemolpymph to the hindgut was suggested for *Pachnoda ephippiata* larvae (38). Both mechanisms are probably not applicable for *Melolontha melolontha* larvae, because in the *Melolontha* midgut hydrogen accumulated just slightly in a restricted region without contact to the hindgut and formate was beyond the detection limit in all gut sections and hemolymph. Moreover, formate did not stimulate methane emission from the hindgut. So far, the reason

for the reduced methane emission of isolated hindguts compared to total larvae remains unclear.

Cell densities and microbial diversity. The intestinal tract of scarabaeid beetle larvae is densely packed with microorganisms (10,38). Cell densities obtained in this study corroborate values determined for humivorous *Pachnoda* species (10,38) and phytophagous *Costelytra zealandica* larvae (1), and confirm the finding that the hindgut of scarabaeid larvae contains several times more cells than the midgut.

Based on 16S rRNA gene T-RFLP fingerprints of three individual larvae, one has to draw the conclusion that the midgut compartment of *Melolontha* larvae does not contain a stable bacterial population, because the number and size of the detected T-RFs were greatly varying. The very low bacterial diversity in 2 investigated individuals (just 1 to 3 T-RFs) is remarkable, since (insect) gastrointestinal systems are usually characterized by a great diversity of microorganisms, which is particularly unraveled when analyses are performed with molecular, culture-independent methods (e.g. (17,27,40,52,53)). The roots with attached soil, obtained from the same meadow as the divot pieces used as food for the larvae, do most probable only weakly represent the food actually consumed by the larvae, assuming that they selectively choose their food. However, the T-RFLP profiles document that this food is probably of a high bacterial diversity, i.e. that the diet is presumably not the reason for the (partially) extremely reduced bacterial diversity in the midgut. Since all investigated larvae looked healthy and were feeding, a harmful microbial infection might also be excluded as an explanation. So far, the only conclusion that can be drawn from the highly variable diversity of the *Melolontha* midgut T-RFLP profiles is, that the bacteria in this compartment probably do not fulfill a particular function for their host, except that they might serve as food.

In contrast to the results obtained with the midgut compartment, the hindgut wall fractions revealed a high bacterial diversity, which was even highly similar among the three investigated individuals (ca. 90% similarity, based on Morisita community similarity indices calculated from the T-RFLP-profiles (13)). Interestingly, the average similarity among the three hindgut wall profiles, obtained from three individual larvae, was significantly higher than the average similarity of corresponding wall and lumen profiles stemming from the same individuals (72%). This finding resemble results recently obtained with soil-feeding *Cubitermes* termites (51), were the bacterial community of homologous gut sections of different species was more similar to each other than that of different gut sections in the same species. The

high similarity of the bacterial hindgut wall community among different *Melolontha* individuals strongly argues for a particular function of this community, the composition of which is probably determined by the special physicochemical conditions at the hindgut wall, e.g. the influx of oxygen up to 100 µm into the gut lumen (this study), or the presence of special attachment structures, i.e. tree-like chitin-structures, which are a typical feature of the hindgut wall of scarabaeid larvae (24) and prevent the attached microorganisms from being washed out with the gut content. The high similarity of the bacterial community at the hindgut wall of individual *M. melolontha* larvae encouraged us to investigate this community in more detail using a 16S rRNA approach, the results of which are presented in the companion study (17).
Compared to the bacterial T-RFLP-profiles (of the *Melolontha* hindguts) but also to archaeal T-RFLP-profiles (obtained with highly comparable PCR-T-RFLP assays) of soil-feeding termites (21) and humivorous *Pachnoda ephippiata* larvae (19), the archaeal diversity was either very low and did not display differences between investigated wall and lumen fractions, indicating the same species composition at hindgut wall and in the lumen. Therefore, the clonal analysis of the archaeal community structure, reported in the companion study (17), was performed with a total *Melolontha* hindgut.
Conclusions. The digestive tract of phytophagous *M. melolontha* larvae is characterized by pronounced axial and radial physicochemical gradients and clear differences in the microbial community composition of its two major compartments (midgut and hindgut) and their subcompartmental fractions (wall and lumen). From a microbiological point of view, microorganisms in the midgut, lacking a stable microbial community, probably do not fulfill a particular symbiotic function, whereas the bacterial community at the hindgut wall, characterized by a high similarity among individual larvae, probably does.

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3.5 Zusammensetzung und räumliche Verteilung mikrobieller Gemeinschaften im Enddarm phytophager Maikäferlarven

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Zusammenfassung: In einer Begleitstudie (Kapitel 3.4) konnten wir zeigen, dass Mittel- und Enddarm phytophager Maikäferlarven hohe Konzentrationen an Acetat sowie eine diverse, aber bislang unbekannte Mikrobiota aufweisen. Besonders interessant war der Befund, dass die bakterielle Gemeinschaft an der Enddarmwand eine hohe Ähnlichkeit zwischen verschiedenen Individuen von Larven aufwies. In dieser Studie haben wir daher die Zusammensetzung und räumliche Verteilung mikrobieller Populationen im Enddarm von Maikäferlarven mit molekularen, kultivierungsunabhängigen Methoden untersucht. Bezogen auf g Frischgewicht war die Enddarmwand ca. 3,5-fach dichter mit Mikroorganismen besiedelt als das Darmlumen. Klonbibliotheken von 16S rRNA-Genen zeigten deutliche Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaften von Darmwand und -lumen. In beiden Fraktionen dominierten Klone mit Verwandtschaft zu den *Clostridiales*, die oft phylogenetische Gruppen entsprechend ihrer Herkunft (Wand oder Lumenfraktion) bildeten. Dies galt in gleicher Weise für Klone mit Verwandtschaft zu den *Bacteroidetes*, die verstärkt an der Darmwand vorkamen. Klone aus den *Actinobacteria*, *Bacillales*, *Lactobacillales* und *γ-Proteobacteria* kamen ausschließlich im Lumen vor, *β-* und *δ-Proteobacteria* ausschließlich an der Darmwand. Die Zusammensetzung der 16S rRNA-Gen Klonbibliotheken, T-RFLP-Analysen und FISH-Analysen mit gruppenspezifischen Oligonukleotidsonden belegten übereinstimmend, dass *Desulfovibrio*-verwandte Bakterien eine abundante Gruppe (10 - 15% aller Bakterien) an der Darmwand der Maikäferlarven darstellen. Dass dies vermutlich ein Charakteristikum aller *M. melolontha* Larven ist, konnten wir über T-RFLP-Analysen von 16S rRNA-Genen und einen PCR-Test, mit dem das für Sulfatreduzierer spezifische *apsA*-Gen nachgewiesen wird, zeigen, die mit Darmextrakten von Maikäferlarven anderer Populationen aus Deutschland und Europa durchgeführt wurden. Die *Archaea*-Population des Maikäferlarven-Enddarms bestand ausschließlich aus *Methanobrevibacter*-Verwandten. Dies bestätigte den Befund der Begleitstudie (Kapitel 3.4), dass Methan nur im Enddarm gebildet wird.

Analysis of microbial community structure and topology in the hindgut of the larva of *Melolontha melolontha* (Coleoptera: Scarabaeidae) reveals an enrichment of *Desulfovibrio*-related bacteria at the hindgut wall

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Abstract

The microbial community in the intestinal tract of soil macroinvertebrates is involved in the transformation of ingested organic matter and thus contributes to the nutrition of its host. In a companion paper (M. Egert, U. Stingl, L. Dyhrberg Bruun, B. Wagner, A. Brune, and M.W. Friedrich, Appl. Environ. Microbiol. X: XXXX – XXXX, 200X) we show that the hindgut of phytophagous European cockchafer (*Melolontha melolontha*) larvae is characterized by high acetate concentrations and a diverse, yet unstudied microbial community. The bacterial community at the hindgut wall was very similar among individual larvae, suggesting a special function of this community. Here we analyzed the microbial community composition and its topological distribution in the hindgut of. *M. melolontha* larvae using cultivation-independent molecular methods. On a fresh weight basis, the hindgut wall was about 3.5-fold more densely populated with microorganisms (DAPI-counts) than the hindgut lumen. 16S rRNA gene clone libraries revealed marked differences between the bacterial community at the hindgut wall and in the hindgut lumen. Both subcompartmental fractions were dominated by clones related to *Clostridiales*, which often formed clusters correlating with their subcompartmental origin. Clones related to *Actinobacteria*, *Bacillales*, *Lactobacillales*, and γ-*Proteobacteria* were restricted to the lumen, and β- and *δ-Proteobacteria* to the hindgut wall. Clone library composition, T-RFLP-analysis and fluorescence in-situ hybridization of whole cells with group-specific oligonucleotide probes revealed that *Desulfovibrio*-related bacteria comprise a significant fraction (10 – 15%) of the bacterial community at the *Melolontha* hindgut wall. T-RFLP analysis of 16S RNA genes and the presence of the adenosine-5´-phosphosulfate reductase gene *apsA* specific for sulfate reducers in gut DNA extracts of lar-

vae from other populations in Europe suggest, that sulfate reducers are generally present at the hindgut wall of *M. melolontha* larvae.

Introduction

The intestinal tracts of many soil arthropods are know to harbor a dense, diverse and active microbial flora, thought to participate in the transformation of ingested organic matter and in host nutrition (15,17,18,43). The insects so far studied best for their interactions with intestinal microorganisms are wood-feeding termites (13,62). Two major functions are commonly attributed to their gut flora, comprising mostly of fermentative bacteria and cellulolytic protozoa, accompanied by homoacetogens and methanogenic archaea (15): A) Depolymerization and fermentative breakdown of (ligno)cellulosic dietary compounds to short-chain fatty acids absorbable by the host and B) improvement of the hosts nitrogen budget by fixation of molecular nitrogen (see also (60)) or mineralization and incorporation of nitrogen-containing excretion products, with nitrogen becoming available for the host again by coprophagy and subsequent digestion of microorganisms.

Due to difficulties to simulate complex microenvironments, culture-independent studies have become indispensable tools to elucidate the composition of microbial communities in insect gut systems, information about which should be linked to gut morphology, physicochemical conditions and the spatial distribution of microorganisms within the gut to gain a better understanding of the digestion processes (14,16). However, besides termites (e.g. (40,72) and studies cited therein), only a few arthropods were studied with molecular methods, including humivorous scarbaeid beetle larvae (*Pachnoda ephippiata*, *Cetoniinae*; (31)). Microbiological studies about phytophagous scarabaeid larvae, comprising several economically important pests (e.g. *Melolontha melolontha* (44), *Costelytra zealandica* (7), *Popillia japonica* (67)) mostly focused on biological pest control, e.g. regarding the use of entomopathogenic fungi (45,74) or potentially enthomopathogenic bacteria (for a review see (47)), e.g. *Serratia* spp. causing amber disease (42) or *Paenibacillus* spp. causing milky disease (66). The natural intestinal microflora of phytophagous scarbaeid beetle larvae was so far only scarcely investigated (5), in particular with molecular methods.

In a companion study (30) we described that the different gut sections of phytophagous *M. melolontha* larvae are characterized by high concentrations of fermentation products and a

dense microbial community. T-RFLP analysis of the bacterial diversity of different gut compartments (midgut and hindgut) and fractions (gut lumen and wall) of individual larvae suggested a relatively transient bacterial community in the midgut. The bacterial community at the hindgut wall, however, displayed a high similarity (ca. 90 %, based on Morisita community similarity indices (27)) among the investigated individuals. Therefore, the major aim of this study was a detailed analysis of the microbial community structure and its spatial distribution in the hindgut compartment of *M. melolontha* larvae, using a combined 16S rRNA gene cloning, T-RFLP, and FISH approach.

All methods consistently revealed that bacteria closely related to *Desulfovibrio* spp. comprise a significant (10 - 15 %) fraction of the bacterial community at the hindgut wall of the investigated larvae. Studies with *M. melolontha* larvae from geographically different sites using T-RFLP analysis and a PCR-assay targeting the adenosine-5'-phosphosulfate-reductase gene *apsA* (32) suggest that *Desulfovibrio* spp. are generally present in *M. melolontha* larvae.

Materials and Methods

Sampling of the investigated *Melolontha melolontha* larvae, dissection, and DNA extraction are explained in detail in the companion paper (30). The origin of the additional *M. melolontha* larvae investigated in this study, stemming from other populations than Obergrombach, Germany, is given in Table 3.

PCR amplification of 16S rRNA and *apsA* genes. For the creation of 16S rRNA gene clone libraries, genes were specifically amplified using the primer combination 27f (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) (28) and 907r (5′-CCG-TCA-ATT-CCT-TTR-AGT-TT-3′) (59) for *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) (37) and Ar915r (5′-GTG-CTC-CCC-CGC-CAA-TTC-CT-3′) (57) for *Archaea*. Standard reaction mixtures and PCR conditions for the amplification of 16S rRNA genes were the same as used for T-RFLP analysis, described in the companion paper (30). The *apsA* genes encoding the α-subunit of the adenosine-5′-phosphosulfate (APS) reductase of sulfate-reducing bacteria (SRB) was specifically amplified using the primer combination APS7-F (5′-GGG-YCT–KTC-CGC-YAT-CAA-YAC-3′) and APS8-R (5′-GCA-CAT-GTC-GAG-GAA-GTC-TTC-3′) (32). The standard reaction mixture contained, in a total volume of 50 µl, 2x PreMix E (Epicentre, Madison, USA), 2 µM of primer APS7-F, 0.5 µM of primer APS8-R (MWG Biotech, Ebersberg, Ger-

many), and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). In addition, 1 µl of a 1:20 dilution of DNA extract was added as template. All reactions were prepared at 4°C in 0.2 ml reaction tubes to avoid non-specific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94°C) block of a Gene Amp 9700 Thermocycler (Applied Biosystems). The standard thermal profile for the amplification of *apsA* genes was as follows: initial denaturation (94°C, 3 min), followed by 35 cycles of denaturation (94°C, 30 s), annealing (52°C, 45 s), and extension (72°C, 90 s). After terminal extension (72°C, 7 min), samples were stored at 4°C until further analysis. Aliquots (5 µl) of 16S rRNA gene amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide. PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany).

T-RFLP analysis. T-RFLP analysis was performed as described in the companion paper (30). To account for pseudo-T-RF formation (29), all clones were checked for their in vitro-T-RF formation pattern. For this purpose, PCR-T-RFLP analysis was performed with 0.5 µl of a 1:10 dilution of clonal M13 product as PCR-template and 16 cycles of amplification. To identify peaks influenced by pseudo-T-RF-formation in T-RFLP profiles of environmental origin (obtained from the hindgut DNA extracts), amplicons were submitted to a digest with mung bean nuclease prior to restriction digest as described in (29), with the following modification: mung bean nuclease digest was terminated by purification with the MinElute PCR purification kit (Qiagen, Hilden, Germany). Subsequently, assignment of T-RFs in the hindgut T-RFLP profiles to clones from the respective libraries was performed considering both, the in vitro T-RF formation pattern of the clones and the alteration of the T-RF patterns by the mung bean nuclease digest.

16S rRNA gene libraries. Bacterial and archaeal 16S rRNA gene amplicons were cloned in *Escherichia coli* JM109 using the pGEM-T Vector System (Promega, Mannheim, Germany) according to the manufacturer's instructions. Randomly selected clones were checked for correct insert size via standard vector-targeted PCR and gel electrophoresis. Sequencing was performed on an ABI Prism 377 DNA sequencer using Big Dye terminator chemistry as specified by the manufacturer (Applied Biosystems), and by the core facility ADIS (Max Planck Institute for Plant Breeding Research, Cologne, Germany). Bacterial clones retrieved from the *Melolontha* hindgut lumen and wall DNA extracts were designated MKEL and MKEW, respectively. Archaeal clones obtained from a total hindgut DNA extract were named MKED.

Phylogenetic analysis. Sequence data were analyzed and trees were constructed using the ARB software package with its database (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München, München, Germany, http://www.arb-home.de). 16S rRNA gene sequences were added to the database and aligned using the Fast Aligner tool (version 1.03). Alignments were corrected manually if necessary. All clonal 16S rRNA gene sequences were compared to sequences in public databases using BLAST (2) and closely related sequences from databases were retrieved and added to the alignment. Trees were constructed using the neighbor-joining algorithm (69) in combination with a base frequency filter (50 to 100% similarity) for *Bacteria* provided with the ARB package. For tree construction, 667 sequence positions (*E. coli* positions 64 - 877) were used. Sequences of species closely related to the gut clones, but significantly shorter than 900 bp were added to the tree using the ARB parsimony tool, which allows the addition of short sequences to phylogenetic trees without changing global tree topologies (56). The terminal sequence positions at the 5'- and 3'-ends of the 16S rRNA gene sequences (ca. 450 bp) were also subjected to a separate treeing analysis ("fractional treeing", (55)). Significant differences in the phylogenetic placement of a fragment pair were considered indicative of chimera formation, and chimeric rRNA gene clones were excluded from further phylogenetic analysis.

Fluorescence in-situ hybridization. FISH with group-specific oligonucleotide probes was performed following a standard protocol (65) with some modifications. Gut samples were homogenized with a sterile glass homogenizer, appropriately diluted with phosphate-buffered saline (PBS) solution and fixed with paraformaldehyde (3%, w/v) at 4°C or ethanol (50%, v/v) at -20°C overnight in the dark. Fixed homogenate (1 ml) was transferred onto white polycarbonate membrane filters (0.2 µm GTTP, Millipore, Eschborn, Germany), washed with PBS (only PFA-fixed samples), air-dried, and stored at –20°C. Prior to FISH analysis, filter pieces were dehydrated using an ethanol series of 50%, 80% and 96% (v/v). Hybridization with triangular filter-pieces was performed for 90 minutes at 46°C using a hybridization buffer with 35% formamide (65). After incubation in washing buffer (20 min, 48°C; (65)), filters were rinsed with distilled water, air-dried and counter-stained with 4,6-diamino-2-phenlyindol (DAPI). For counting, filters were embedded in Citifluor solution (AF1, Citifluor Ltd., London, UK). Cells were counted by epifluorescence microscopy using an ocular grid. At 1000-fold magnification at least 1000 cells were counted for each sample. Only cells which had a positive DAPI and probe signal were counted. Bacterial cells were detected using

an equimolar mixture of the probes EUBI – III (22), archaeal cells were targeted with a mixture of ARC344 (68) and ARCH915 (77). To detect sulfate-reducing bacteria probe SRB385 (3) and a probe specifically targeting 16S rRNA sequences of *Desulfovibrio* spp. detected in the cockchafer larvae (DSV698-MK) were used. The sequence of probe DSV698-MK was 5´-GTT-CCT-CC**T**-GAT-**C**TC-TAC-GG-3´ with bold letters indicating modified positions compared to probe DSV698 (58), to which most of the *Desulfovibrio*-related sequences from the *Melolontha* gut had 2 mismatches. Based on the sequence data of the hindgut lumen and wall library, most (9 out of 11) *Desulfovibrio*-related bacteria were expected to be detected by the DSV698-MK probe, one clonal sequence was too short for a prediction, one had two mismatches. All other remaining clones (153) displayed at least 2 mismatches with probe DSV698-MK. Unfortunately, no clonal sequence perfectly matched the SRB385 probe. However, only the *Desulfovibrio*-related clones had one mismatch at the very end (5´) of the target sequence (T instead of C) while all other clones with one mismatch (6 clones in the hindgut wall and 26 in the lumen library, all related to *Clostridiales*) had a mismatch located in the center of the target sequence.

All probes were labeled with CY3, probe sequences and full-names *sensu* (1) are available via probeBase (http://www.microbial-ecology.de/probebase; (53)).

Estimation of species richness and statistical analyses. Bacterial species richness in the hindgut lumen and hindgut wall clone libraries was estimated using Chao1 as nonparametric indicator (19), calculated with EstimateS (version 5.0.1; R. Colwell, University of Connecticut [http://viceroy.eeb.uconn.edu/estimates]) as described in (41). For this purpose, a "species" was defined as a group of 16S rRNA gene clones with ≥ 97% sequence similarity (76). Differences between samples (in FISH cell counts) were checked for statistical significance with non-parametric Mann-Whitney tests using SYSTAT 10.0 (SPSS Inc., Chicago, USA).

Sequence data. Bacterial 16S rRNA gene sequences of clones from the hindgut lumen of the *M. melolontha* larva (MKEL-clones) and the hindgut wall (MKEW-clones) are accessible under…, respectively. 3 representative archaeal sequences (MKED-clones), retrieved from a total hindgut, are accessible under….

Results

16S rRNA gene clone libaries. Two separate clone libraries were generated from 16S rRNA gene fragments amplified from a hindgut lumen and a hindgut wall DNA extract of a *M. melolontha* larva with *Bacteria*-specific primer pairs. In addition, an archaeal 16S rRNA gene clone library was established from a DNA extract of a total *M. melolontha* hindgut. From all clone libraries, randomly selected clones were sequenced and phylogenetically analyzed. Chimeric sequences were identified using fractional treeing and excluded from further analyses.

Phylogenetic analysis revealed that all clones were affiliated with known taxa of the *Bacteria* (Fig. 1 to 3, Table 1) and *Archaea* (not shown). Within all taxa, many clones obtained in this study were clustering with cultured representatives and/or molecular isolates from the intestinal tract of higher animals (ruminants, rodents, pigs), humans and other insects, in particular termites and cetoniid beetle larvae.

Bacterial clones obtained from the hindgut lumen (n = 90) could be assigned to 7 distinct phylogenetic groups (Fig. 1 to 3). Most lumen clones (47%) were affiliated with *Clostridiales*, (Fig. 1). Several of them could be assigned to the clostridial clusters IV, IX, XIVa & b and XVI *sensu* Collins et al. (20), however, many were grouping (together with other intestinal clones or isolates) outside these clusters. 24 lumen clones were clustering with *Turicibacter sanguinis* (92 – 97% sequence identity), a strictly anaerobic gram-positive bacterium isolated from a human blood-sample (10). Members of *Actinobacteria*, *Bacillales*, *Lactobacillales*, γ-*Proteobacteria* and the *Bacteroidetes* were less frequently recovered, however, members of the first 4 groups occurred exclusively in the hindgut lumen clone library (Fig. 2 and 3).

The 74 bacterial clones from the hindgut wall sample fell into 5 distinct phylogenetic groups (Fig. 1 to 3). By far most clones of the gut wall (61%) were affiliated with *Clostridiales*, partly assignable to the clostridial clusters I, IV, XI and XIVa, partly grouping outside known clusters (Fig. 1). 12 wall clones were related to members of the *Bacteroidetes*, but in contrast to the lumen clones, none of them clustered with *Dysgonomonas* species. *Turicibacter*-related clones and clones grouping with the β-group of *Proteobacteria* were of lower frequency.

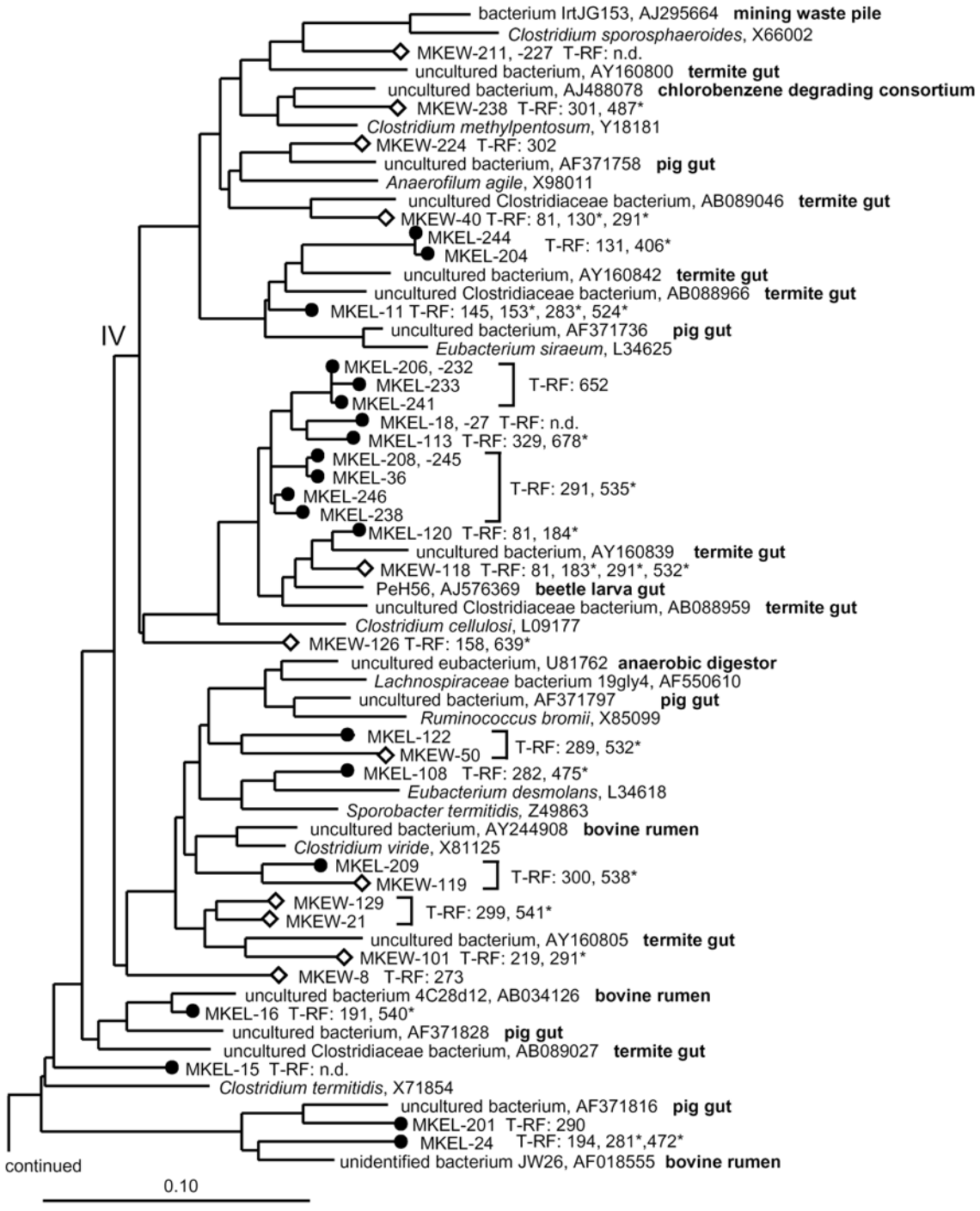

Figure 1. Phylogenetic tree showing the position of 16S rRNA gene sequences affiliated with *Clostridiales*, recovered from the hindgut lumen (black dots) and the hindgut wall (open rhombs) of a *M. melolontha* larva. Scale bar represents 10% sequence difference. Accession number of reference sequences are indicated. Species used as outgroup were *Thermus thermophilus* (M26923), *Fervidobacterium gondwanense* (Z4917) and *Thermotoga maritima* (M21774). Roman numerals indicate clostridial subgroups sensu (20). Lengths of T-RFs result from in vitro digestion of clonal 16S rRNA gene amplicons with *Msp*I, pseudo-T-RFs (29) are marked with asterisks. n.d.: T-RF formation in vitro not determinable.

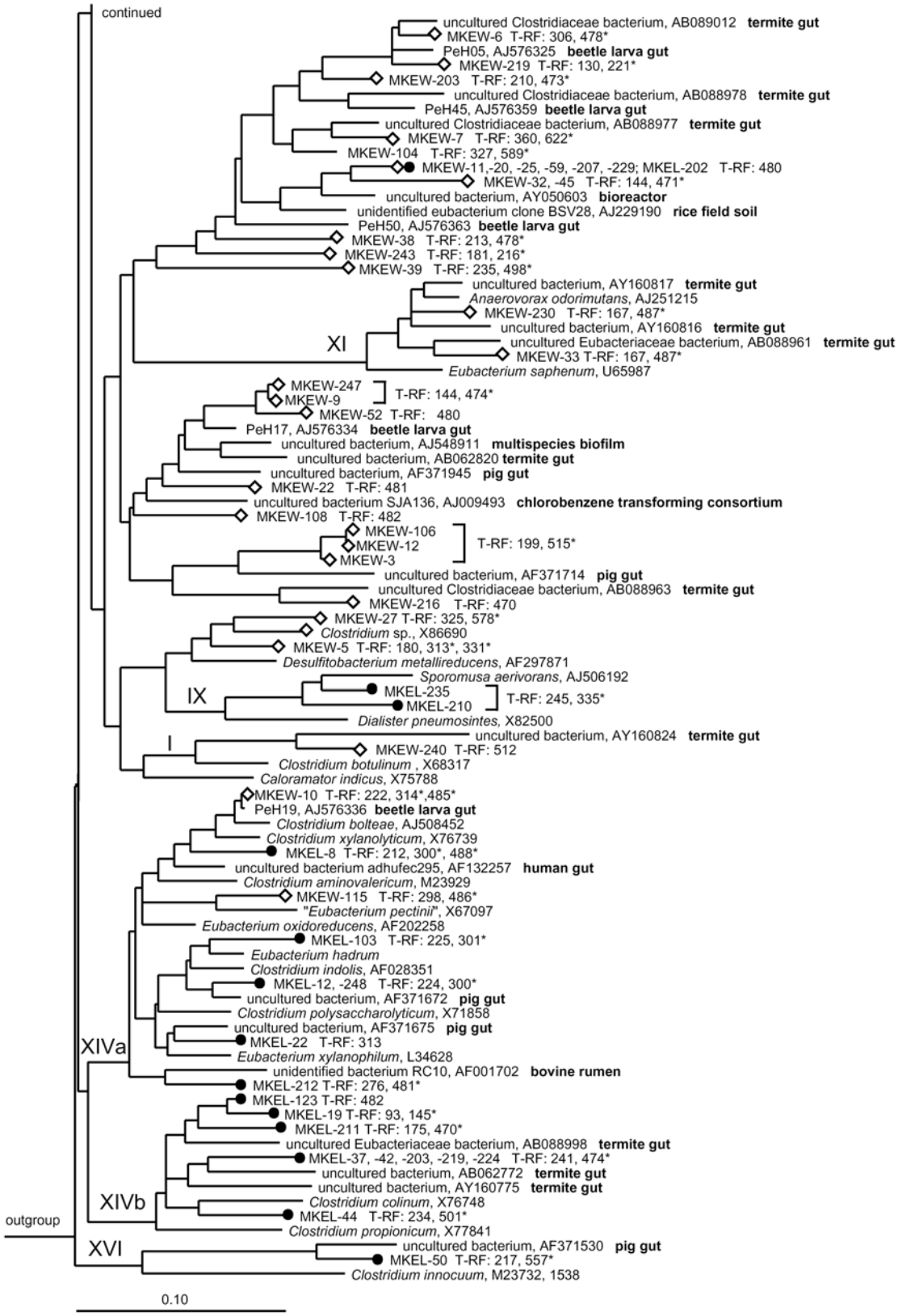

Figure 1. continued

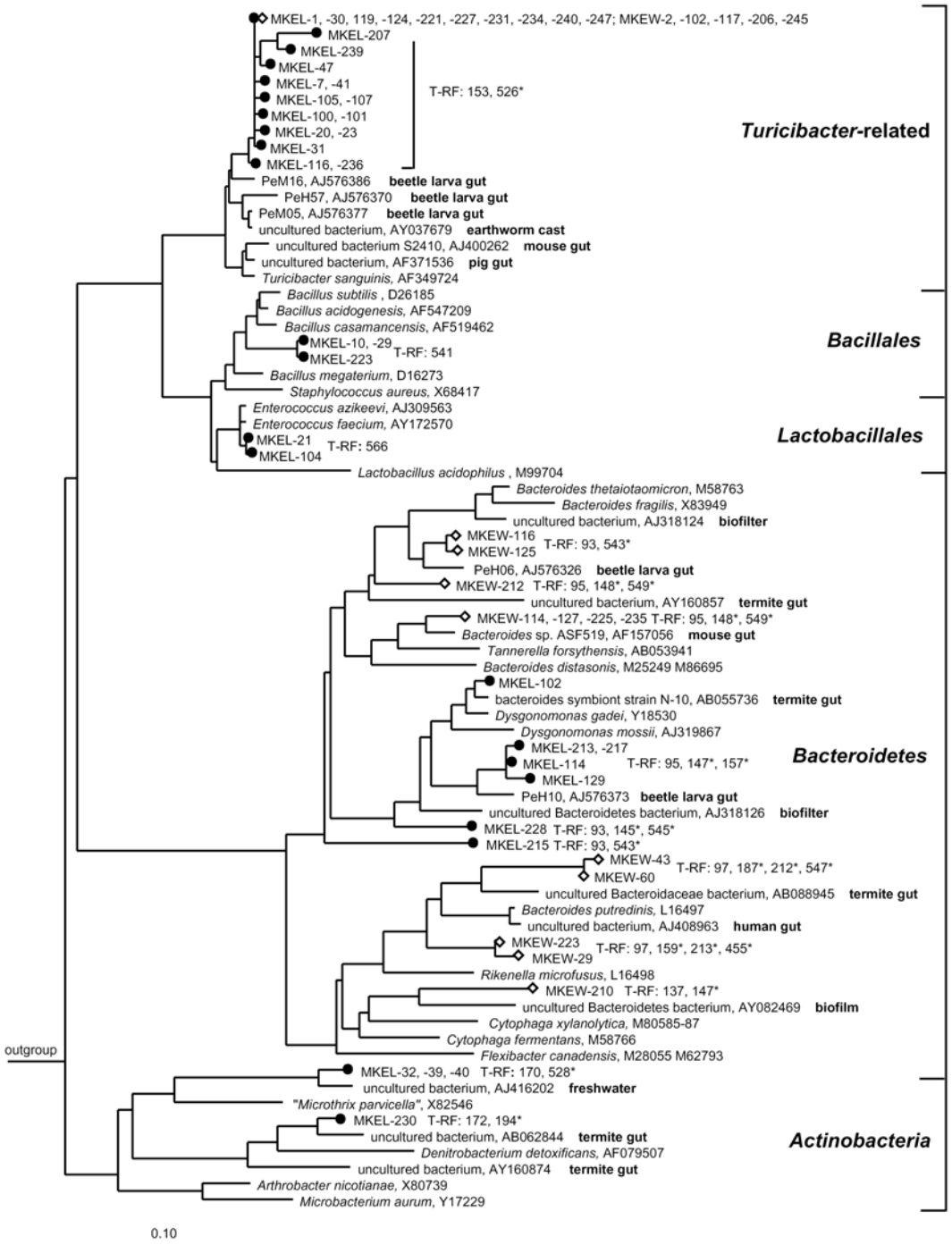

Figure 2. Phylogenetic tree showing the position of 16S rRNA gene sequences affiliated with *Turicibacter* spp., *Bacillales*, *Lactobacillales*, *Bacteroidetes*, and *Actinobacteria*, recovered from the hindgut lumen (black dots) and the hindgut wall (open rhombs) of a *M. melolontha* larva. Scale bar represents 10% sequence difference. Accession number of reference sequences are indicated. Species used as outgroup were *Thermus thermophilus* (M26923), *Fervidobacterium gondwanense* (Z4917) and *Thermotoga maritima* (M21774). Lengths of T-RFs result from in vitro digestion of clonal 16S rRNA gene amplicons with *Msp*I, pseudo-T-RFs (29) are marked with asterisks.

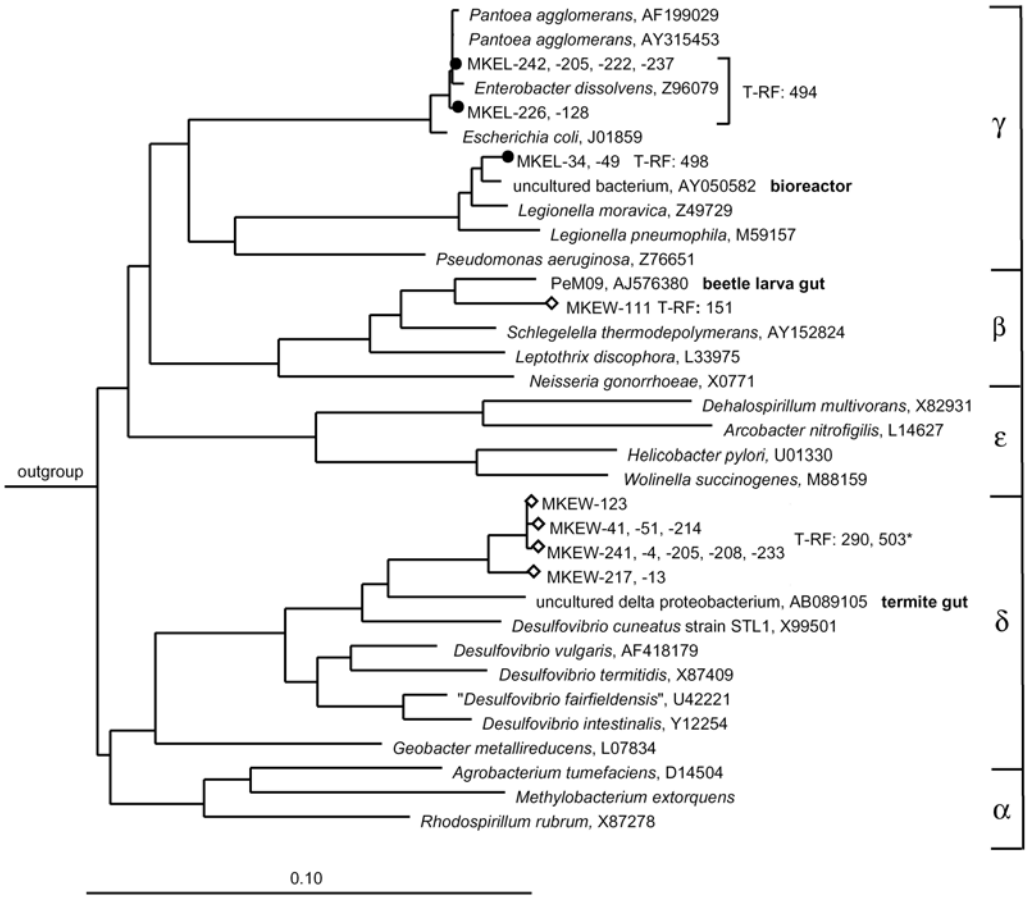

Figure 3. Phylogenetic tree showing the position of 16S rRNA gene sequences affiliated with the 5 subclasses of *Proteobacteria*, recovered from the hindgut lumen (black dots) and the hindgut wall (open rhombs) of a *M. melolontha* larva. Scale bar represents 10% sequence difference. Accession number of reference sequences are indicated. Species used as outgroup were *Thermus thermophilus* (M26923), *Fervidobacterium gondwanense* (Z4917) and *Thermotoga maritima* (M21774). Lengths of T-RFs result from in vitro digestion of clonal 16S rRNA gene amplicons with *Msp*I, pseudo-T-RFs (29) are marked with asterisks.

The most prominent difference between the hindgut lumen and wall libraries was the occurrence of clones affiliated with the genus *Desulfovibrio* of the *δ-Proteobacteria* exclusively in the gut wall library, comprising 15% of all hindgut wall clones (Fig. 3). These clones were closest related to an uncultured bacterium (Rs-K75) from the gut of the wood-feeding termite *Reticulitermes speratus* (88.6 – 90.4 % sequence similarity), their next cultured relative was *Desulfovibrio cuneatus* strain STL1 (85.6 – 88.5% sequence similarity), a psychrotolerant *Desulfovibrio*-species isolated from an oxic freshwater sediment (71).

TABLE 1: Relative abundance (%) of major phylogenetic groups in midgut and hindgut of a *M. melolontha* larva, based on the frequencies of 16S rRNA genes in clone libraries (n = 90 [midgut] and 74 [hindgut]) and on T-RFLP analysis. For explanation of T-RFLP-based frequency ranges, see text. n.d. = not detected; rel. = related

Phylogenetic group	**Hindgut lumen**		**Hindgut wall**	
	Clone library	**T-RFLP**	**Clone library**	**T-RFLP**
Actinobacteria	4.4	1.0	n.d.	
Bacillales	3.3	1.4	n.d.	
Lactobacillales	2.2	0.7	n.d.	
Turicibacter-rel.	26.7	15.3	6.8	1.0 - 4.9
Clostridiales	46.7	58.9 - 60.1	60.8	38.1 - 52.5
Bacteroidetes	7.8	3.1 - 4.3	16.2	22.3
β-*Proteobacteria*	n.d.		1.4	0 - 3.9
γ-*Proteobacteria*	8.9	2.8	n.d.	
δ-Proteobacteria	n.d.		14.9	0.8 - 15.3

By using an arbitrarily defined limit of 97% sequence similarity the 16S rRNA gene clones in the hindgut lumen and wall libraries could be grouped into 39 or 45 different species, respectively. The total species richness of both hindgut samples was estimated using the data from the clone libraries and Chao1 as non-parametric richness estimator. The estimated number of species were 63 for the lumen compartment and 127 for the hindgut wall, which means that the clonal approach detected about two-third of the species potentially present in the hindgut lumen and more than one-third of the species present at the hindgut wall of the investigated larva. Since the corresponding 95% confidence intervals were 42 and 110 for the lumen sample and 73 and 252 for the wall sample (calculated for the lowest number of clones shared, i.e. 74), species richness at the hindgut wall can not be regarded to be significantly higher than in the hindgut lumen.

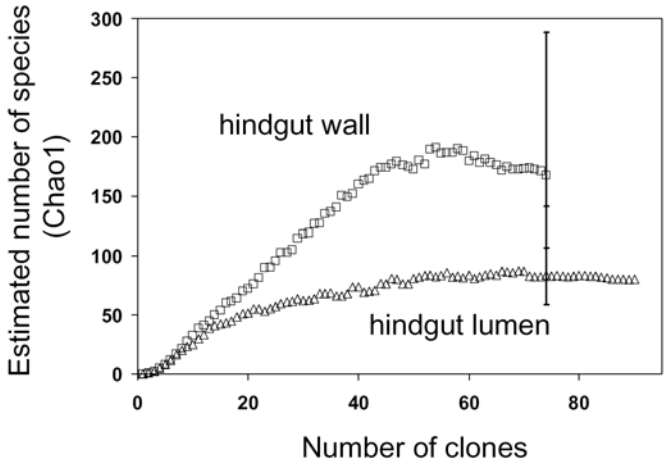

Figure 4. Estimation curves (Chao1) for the potential number of bacterial species at the hindgut wall and in the hindgut lumen of a *M. melolontha* larva, based on the composition of 16S rRNA gene clone libraries obtained from a hindgut wall (n = 74 clones) and a hindgut lumen (n = 90 clones) sample. Error bars for the lowest number of shared clones (74) represent 95% confidence intervals.

However, as the estimation curves for both investigated samples were noticeably leveling off towards their ends (Fig. 4), one can assume that the estimation of species richness is quite reliable, which in turn allows careful speculations that species richness at the hindgut wall might actually be higher than in the hindgut lumen. However, probably several hundreds of clones more have to be analyzed to claim this with 95% confidence.

For the archaeal clone library, 30 randomly selected clones from a total hindgut of a *M. melolontha* larva were sequenced and phylogenetically analyzed. However, all clones were virtually identical in sequence (≥ 99.8% sequence identity) and clustering within the methanogenic Euryarchaeota (*Methanobacteriaceae*; not shown). They were closely related (~ 99.5% sequence similarity) to clones recently obtained from the intestinal tract of humivorous *Pachnoda ephippiata* larvae (e.g. PeHAr10 and PeMAr14; (31)), which in turn clustered with clones from the gut of the soil-feeding termite *Cubitermes orthognathus*. *Methanobrevibacter arboriphilus* (AB065294) was found to be the closest cultured relative (~ 97.8 % sequence identity).

T-RFLP analysis. Structure and diversity of the bacterial communities in the hindgut lumen and at the hindgut wall of the investigated larva and of the archaeal community in a total *Melolontha* hindgut were also assessed directly by T-RFLP analysis, as reported in the companion paper (30). In silico determination of the expected size of T-RFs allowed us to assign T-RFs

in the fingerprints (profiles “hindgut lumen 1” and “hindgut wall 1” in Fig. 3 and “total hindgut” in Fig. 4 of the companion paper (30)) to distinct phylogenetic groups (Fig. 5). To account for the bias of pseudo-T-RF formation (29), we also considered the in vitro T-RF formation pattern of the clones and the effect of a mung bean nuclease treatment prior to T-RFLP analysis on the environmental profiles, when T-RFs were assigned to phylogenetic groups.

Since some T-RFs were shared by more than one phylogenetic group (Fig. 5), the T-RFLP-based frequency of these groups can be expressed as frequency ranges only. Minimum frequencies were calculated by considering only those T-RFs that were unique for a certain group, whereas for maximum frequencies those T-RFs shared with others were also taken into account.

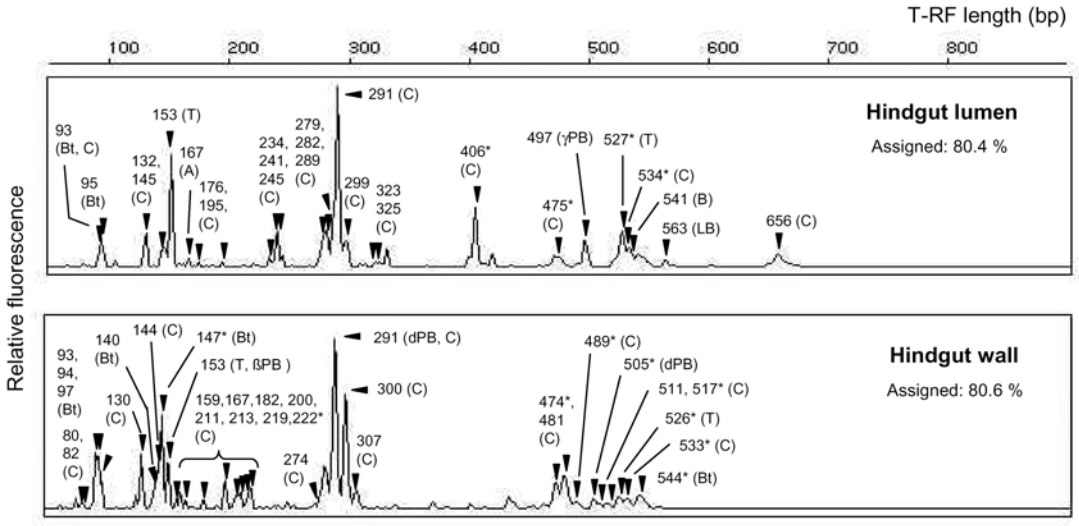

Figure 5. T-RFLP profiles of bacterial 16S rRNA genes amplified from a hindgut lumen and a hindgut wall sample of a *M. melolontha* larva. *Msp*I was used for restriction digest. Assignable T-RFs are marked with triangles. Asterisks indicate T-RFs probably influenced by pseudo-T-RF formation (29). Assignable groups are given in parentheses with the following abbreviations: A: *Actinobacteria*, B: *Bacillales*, Bt: *Bacteroidetes*, C: *Clostridiales*, LB: *Lactobacillales*, βPB: β-*Proteobacteria*, γPB: γ-*Proteobacteria*, δPB: δ-*Proteobacteria*, T: *Turicibacter*-related.

In both bacterial T-RFLP profiles from the *Melolontha* hindgut the assignable T-RFs represented more than 80% of the total peak height (Fig. 5). All phylogenetic groups present in the two clone libraries were found to be represented by T-RFs in the respective profiles (Table 1). The differences in the relative gene frequencies when based on T-RFLP or clonal data can be explained by the limited number of analyzed clones. However, T-RFLP based gene frequencies in general corroborated the trends expressed by clone libraries, e.g. that 16S rRNA gene

sequences affiliated with *Clostridiales* are (relatively) dominating the amplicon pools obtained from both hindgut fractions or that *Turicibater*-related genes are relatively more abundant in the hindgut lumen than in the hindgut wall fraction. Unfortunately, the *Desulfovibrio*-related clones shared a 290 bp T-RF with a clostridial clone (MKEW-50). However, since the hindgut wall library contained 11 *Desulfovibrio*-related clones and just one clostridial clone with a *Msp*I restriction site yielding a 290 bp T-RF, the corresponding T-RF in the hindgut wall profile is apparently largely assignable to the *Desulfovibrio*-related sequences. Therefore, the relative frequency of their genes in the amplicon pool is probably close to 15% (Table 1), which is in very good accordance with the clone library data. Accordingly, also in hindgut wall T-RFLP profiles of two other larvae, presented in the companion paper (Fig. 2 in (30)), a dominant 291 bp T-RF occurred making up 12 - 15% of the total electropherogram peak height.

All archaeal T-RFLP profiles from the hindgut of *Melolontha* larvae, irrespective if they were obtained from total hindguts or guts separated into wall and lumen fractions, displayed a uniform pattern, consisting almost exclusively of a dominant 67 bp and a smaller 167 bp T-RF (Fig. 3 in (30)). Both T-RFs can be assigned to the *Methanobrevibacter*-related clones in the clone library, which displayed a 67 bp T-RF and a 167 bp pseudo-T-RF, as already the closely-related clones from the hindgut of *Pachnoda* larva did (31).

FISH results. Two larvae were investigated for the abundance of bacterial and archaeal cells in total guts using domain-specific oligonucleotide probes. In these larvae, 36.2 and 47.0% of all DAPI-stained cells from the midgut and 61.5 and 73.5% of all DAPI-stained cells from the hindgut were also detected with a mixture of the three EUB probes targeting all bacteria. Using a mixture of two probes targeting *Archaea,* no positive signals could be obtained from the two midgut samples. In case of the hindguts, 0.1 - 0.2% of all DAPI-stained cells showed positive probe signals, which results in a ratio of 2 - 3 archaeal cells per 1000 bacterial cells. Interestingly, archaeal signals were only detected when cells were fixed with ethanol but not with PFA as fixing agent.

The spatial distribution of cells in the hindgut was studied with three individual *M. melolontha* larvae, whose isolated hindguts were each separated into a lumen and wall fraction (Table 2).

TABLE 2: Absolute cell numbers and percental frequencies of different target group obtained by DAPI-staining and FISH-counts with group-specific oligonucleotide probes. Values represent mean ± standard error, based on three individual *M. melolontha* hindguts, each separated into wall and lumen fraction. Means within rows followed by the same letter are not significantly ($P < 0.05$) different.

Stain / Probes	Hindgut lumen	Hindgut wall
DAPI [counts g Fwt^{-1}]	$0.95 \pm 0.37 \times 10^{10}$ a	$3.2 \pm 0.7 \times 10^{10}$ b
EUB 338 I-III [% DAPI counts]	60.4 ± 3.1 a	53.7 ± 4.8 a
ARC344/ARCH915 [% EUB counts]	0.2 ± 0.1 a	1.3 ± 0.4 b
SRB385 [% EUB counts]	0.7 ± 0.1 a	9.4 ± 1.9 b
DSV698-MK [% EUB counts]	1.1 ± 0.9 a	8.7 ± 2.0 b

Based on DAPI-counts and fresh weight, cell density at the hindgut wall of *Melolontha* larvae was approximately 3.5 time higher than in the hindgut lumen. The fraction of DAPI-stained cells which also showed a positive signal when hybridized with the *Bacteria*-specific EUB probes was not significantly different between the lumen and wall fractions (54 - 60%). Approximately 7 times more archaeal cells were detected at the hindgut wall than in the lumen. On average, 13 archaeal cells were counted per 1000 bacterial cells at the hindgut walls, which is considerably more than the counts found with total hindguts.

The abundance of sulfate-reducing bacteria (SRB) in the hindgut lumen at the hindgut wall was investigated using the SRB385 probe, targeting SRB affiliated with δ-*Proteobacteria* but also some gram-positive bacteria (probeBase; http://www.microbial-ecology.de/probebase) and a modified DSV698 probe, targeting *Desulfovibrio* spp. of the *Melolontha* hindgut. The obtained results (Table 2) suggest that the different location of mismatches (see Material and Methods sections) allowed a specific hybridization of the *Desulfovibrio*-related sequences also with probe SRB385 while it obviously prevented an interfering staining of the bacteria related to *Clostridiales*.

On an average of three individual hindgut wall and lumen samples, respectively, hybridization with the DSV698-MK probe stained 8.7% of all bacterial cells. Assuming that this probe detects just 90% of all *Desulfovibrio*-related sequences, the fraction of these bacteria at the hindgut wall can be estimated to be ca. 9.5% of all bacteria. This assumption was perfectly matched by the results obtained with the SRB385 probe (Table 2). Compared to the hindgut lumen, 8 - 13 times more *Desulfovibrio*-related bacteria were detected at the hindgut wall. In all, FISH results indicate that ca. 9 - 10% off all bacterial cells at the hindgut wall of the investigated *Melolontha* larvae are bacteria related to *Desulfovibrio* spp., which corroborates the results obtained by cloning and T-RFLP analysis (Table 1).

Presence of *Desulfovibrio* spp. in *M. melolontha* populations of geographically different origin. To address the question, if the relatively high abundance of *Desulfovibrio* species at the hindgut wall is a common feature of *M. melolontha* larvae in general (or perhaps a population or food-dependent phenomenon), we analyzed *M. melolontha* larvae from four other populations of geographically different origin (Table 3).

TABLE 3: Relative frequencies (%) of ~ 291 bp T-RFs in bacterial 16S rRNA gene profiles obtained from hindgut lumen and wall DNA extracts of *M. melolontha* larvae from different populations, and results of PCRs targeting the APS-reductase gene *apsA* in the same DNA extracts. # = number of individual larva.

Origin of Larvae		**Hindgut lumen**		**Hindgut wall**	
		291 bp T-RF (%)	***apsA*-PCR**	**291 bp T-RF (%)**	***apsA*-PCR**
Switzerland [a]	#1	3.8	negative	13.5	positive
	#2	3.3		8.2	
Italy [b]	#1	22.2		16.6	
	#2	14.2		18.7	
Germany [c]	#1	3.5		21.0	
	#2	6.8		14.3	
Denmark [d]	#1	6.4		7.2	

[a] meadow near Lungern, canton Oberwalden, Switzerland
[b] from Schlanders, South Tyrol, Italy
[c] wine-yard near Endingen-Kiechlingsbergen, federal state Baden-Württemberg, Germany
[d] fir plantation near Hadsund, Northern Jylland, Denmark

These larvae had been kept in a Sphagnum-substrate at ~15°C and had been fed with carrots. T-RFLP analysis of 16S rRNA genes, performed with DNA extracts of total midguts (not shown), hindgut lumen and hindgut wall fractions, revealed the presence of a ~ 291 bp T-RF, which represented the *Desulfovibrio*-related bacteria in the hindgut wall profiles of the Obergrombach larvae, but to a minor extent also some *Clostridia*, in almost all investigated samples (Table 3). However, with a PCR system specifically targeting the functional marker gene *apsA* of sulfate reducing bacteria, positive signals could be obtained only from the hindgut wall but not from the hindgut lumen or midgut DNA extracts. This outcome was unexpected, because the FISH analysis (but not the clone libraries) performed with the larvae from Obergrombach indicated the presence of a few sulfate reducers also in the hindgut lumen. However, it can not be excluded that positive *apsA* PCR-results could have obtained also from the hindgut lumen DNA extracts with an increased PCR-cycle number.

Nevertheless, the presence of a ~291 bp *Msp*I T-RF (with relative frequencies comparable to the those obtained for the larvae from Obergrombach) in the T-RFLP profiles in combination with positive *apsA* PCR-results from the hindgut wall DNA extracts only, are good indications that the *Desulfovibrio*-related bacteria detected in this study might form a significant proportion of the bacterial hindgut wall community of all *M. melolontha* larvae, irrespective of origin and food. The results do not rule out the existence of sulfate reducers in the hindgut lumen, but they are a strong indication for an enrichment at the hindgut wall.

Discussion

This is the first comprehensive report about the subcompartmental topology (gut wall vs. lumen) of microbial communities in an arthropod gut system and the first report which undoubtedly proves an enrichment of sulfate reducing bacteria at the hindgut wall of an arthropod. In a companion study (30) we demonstrated that the intestinal tract of phytophagous *Melolontha melolontha* larvae is characterized by pronounced radial and axial gradients regarding physicochemical conditions and microbial community composition. Compared to the midgut compartment, which obviously lacks a specific flora, the hindgut of *Melolontha* larvae was more densely populated with microorganisms, forming a diverse but stable community particularly at the hindgut wall, which was very similar among different individual larvae. (30). In this study we analyzed the bacterial and archaeal community structure at the hindgut

wall and in the hindgut lumen of *M. melolontha* larvae with cultivation-independent molecular techniques. Our results show that the bacterial (but not the archaeal) community at the hindgut wall and in the lumen is markedly different, with the enrichment of *Desulfovibrio*-related bacteria at the hindgut wall being one of the most prominent differences.

Because in highly diverse microbial communities a cloning approach can be biased by undersampling (72), we also employed T-RFLP analysis to corroborate the results obtained with the 16S rRNA gene clone libraries and we used FISH with group-specific oligonucleotide probes as a PCR-independent method to account for possible PCR-biases (82).

Bacterial community. The high degree of bacterial diversity found in the hindgut of *Melolontha* larvae is typical for many gut ecosystems studied so far with molecular techniques (e.g. (31,40,51,54,61,73,78,79)). All clones obtained in this study were affiliated with well-described phylogenetic groups, commonly found in intestinal systems (*Actinobacteria*, *Bacillales*, *Bacteroidetes*, *Clostridiales, Lactobacillales*, *Proteobacteria*; see studies cited above) and also relatives of *Turicibacter sanguinis* (10) have already been cloned from intestinal systems (31,70). On the contrary, clones related to spirochetes, possibly accounting for up to 50% of all bacteria in some termites, e.g. (40,64), were not detected at all.

Throughout all detected taxa, clones isolated from the *Melolontha* hindgut were frequently clustering with clonal sequences retrieved from the intestinal tract of other animals, ranging from other insects (soil-feeding termites (73) and beetle larvae (31), wood-feeding termites (40,63) to pigs (51), ruminants (79,80,84) and humans (39,78). The phylogenetic affiliations with other gut microorganisms, including isolates, are a strong argument, that the majority of the *Melolontha* hindgut flora is gut-specific, and comprises not merely of food-derived microorganisms flourishing under the favorable conditions of a gut system. The unstable, transient character of the microbial community in the midgut compartment, which partly showed an extremely reduced diversity, corroborates the assumption, that the bulk of the hindgut flora is probably not stemming from ingested food. However, clones related to *Pantoea agglomerans* (Fig. 3), occurring exclusively in the lumen fraction, probably constitute an exception. These clones displayed a ca. 495 bp T-RF with *Msp*I as restriction enzyme and clones virtually identical to those obtained from the hindgut lumen could be identified to cause the 495 bp T-RF in the midgut T-RFLP profiles with the very reduced diversity (Fig. 2 in (30), (M. Egert, L. Dyhrberg Bruun, B. Wagner and M.W. Friedrich, unpublished results). Since also the 16S rRNA gene T-RFLP profiles of roots with attached soil displayed a 495 bp T-RF and *P. ag-*

glomerans is known to be distributed ubiquitously (35) including plant surfaces (4), relatives of *P. agglomerans* might stem from ingested food, i.e. grass roots. However, *P. agglomerans* may be permanently associated with the gut of insects, e.g. of western flower thrips (23), and it can perform important functions there, e.g. production of hormone precursors, even it if is acquired from ingested food (25,26).

As described in the companion study (30), acetate was by far the most abundant short chain fatty acid in the *Melolontha* hindgut. Clones closely related to known cultivated acetogenic bacteria, e.g. of the genera *Clostridium* or *Eubacterium*, corroborate this finding. A great diversity of *Clostridiales*-related intestinal clones was also observed in molecular diversity studies of termites (40,73), domesticated (79,84) and wild ruminants (61), pigs (51), and humans (78). A pronounced decrease of glucose concentration from mid- to hindgut (30) indicates that glucose is one of the major substrates for fermentations in the *Melolontha* hindgut. However, the great diversity of clones related to *Clostridiales* and *Bacteroidetes* indicates, that also structural plant polysaccharides (cellulose, hemicellulose) and proteins may be hydrolyzed in the *Melolontha* hindgut, and the resulting monomers subsequently fermented. The *Melolontha* midgut displays pronounced protease activities (83), and aminoacids liberated from proteins in the midgut might also directly serve as a substrate in hindgut fermentations. In contrast to humivorous *Pachnoda ephippiata* larvae (50), the lactate concentration in the *Melolontha* hindgut was almost neglectable, which is corroborated by the low frequency of clones related to *Lactobacillales* (2.2% compared to 30.8% in a *Pachnoda* hindgut 16S rRNA gene library; (31)). However, lactate is also the main metabolic product of *Turicibacter sanguinis* (10), to which almost 18% of all 164 investigated clones (mostly lumen clones) were closely related to (92 - 97% sequence identity). Most likely the *Turicibacter* spp. from the *Melolontha* gut have different physiological properties than their closest cultured relative or the absence of lactate indicates, that it is utilized in other processes (see below).

The microbial communities in the hindgut lumen and at the hindgut wall differed considerably in their composition, which suggests that they perform different functions on a microscale. Differences in the bacterial community composition of hindgut wall and lumen have also been reported for the wood-feeding termite *Mastotermes darwiniensis* (6). Total cell counts revealed, that on a fresh weight basis, the hindgut wall compartment comprised approximately 3.5 x more cells than the hindgut lumen. At the hindgut wall microbial cells form a thick biofilm (M. Egert, L. Dyhrberg Bruun, A. Brune, and M.W. Friedrich, unpublished

results), attached to tree-like chitin structures, which are characteristic features of the hindgut of scarabaeid larvae (38). Clones related to *Actinobacteria*, *Bacillales*, *Lactobacillales*, and γ-*Proteobacteria* were exclusively found in the lumen fraction. Clones grouping among *Bacteroidetes* were detected in both fractions, however, all lumen clones were closely related to *Dysgonomonas* species while the wall clones were clustering separately with *Bacteroides* and *Cytophaga* species. Also in case of the *Clostridiales*, wall and lumen clones were grouping according to their subcompartmental origin, with the clostridial clusters IV and XIVa and a cluster around *Sporobacter termitidis* being exceptions, were clones from both compartments were mixing. One factor determining the community composition of hindgut wall and lumen might be oxygen, which penetrates ca. 100 µm deep into the gut lumen (30). The most pronounced difference between the hindgut lumen and wall communities, however, was the abundant occurrence of *Desulfovibrio*-like bacteria at the hindgut wall, determined by both PCR-dependent and independent methods to comprise a significant fraction (ca. 10 - 15%) of the bacterial community there. T-RFLP-profiles and results of a PCR-assay targeting the *apsA* gene of sulfate-reducing bacteria (32), obtained with larvae stemming from another German population and from northern Italy, Switzerland, and Denmark, indicate that this is a common feature of *Melolontha melolontha* larvae.

Sulfate reducing bacteria of the genus *Desulfovibrio* are known inhabitants of gastrointestinal tracts, e.g. of termites (e.g. (34,81)) and higher animals including humans (e.g. (24,52)). However, to our knowledge, a comparable dominance (10 - 15% off all bacteria) at an arthropod gut wall has so far not been reported before. With a FISH approach, Berchtold and coworkers (6) reported an enrichment of sulfate reducers at the hindgut wall of *Mastotermes darwiniensis* termites, but they only used SRB385-probes, know to target many non-sulfate-reducing bacteria (for a specificity check see http://www.microbial-ecology.de/probebase; (53)). In this study, however, we used probe DSV698-MK, designed to specifically detect the *Desulfovibrio* spp. at the *Melolontha* hindgut wall and to which all other clonal sequences analyzed in this study displayed at least 2 mismatches. *Desulfovibrios* have a well known capability to use molecular O_2 as electron acceptor (21), i.e. they are microaerotolerant. Based on that and other traits of *Desulfovibrio* species isolated from termites, Kuhnigk and coworkers (48) proposed some feasible functions for intestinal SRB, from which an insect (termite) host would benefit: a) incomplete oxidation of fermentation products (lactate, ethanol) to acetate, the preferred product for uptake by the host; b) removal of inflowing O_2 via complete

oxidation of H_2 or formate, which are of no use for the host (the removal of O_2 would prevent the gut acetate pool from oxidation, the removal of hydrogen shifts the fermentation balance of sugars to more acetate); c) oxidation of sulfide to prevent toxic effects of H_2S. d) dinitrogen fixation to improve the nitrogen balance of the host. The host benefits from the fact that, compared to several other aerobic and facultatively anaerobic bacteria as well as several aerobic H_2 oxidizers, *Desulfovibrio* species can not oxidize acetate.

The location of the *Desulfovibrio*-related bacteria at the hindgut wall of *Melolontha* larvae suggests, that they might face oxygen, which penetrates up to 100 µm deep into the hindgut lumen (30). However, the biofilm at the hindgut wall appears to be thicker than 100 µm (M. Egert, L. Dyhrberg Bruun, A. Brune, and M.W. Friedrich, unpublished results), so that a more detailed spatial analysis of the orientation of the SRB in the biofilm is needed to determine their exposition to oxygen. The high acetate and low lactate concentration (formate and ethanol were not discovered at all) and hydrogen concentrations beyond the detection limit (despite the relative dominance of *Clostridiales*-related clones) in the hindgut (30) are in favor of the above proposed functions of the *Desulfovibrio*-like bacteria. However, hydrogen scavenging might also be carried out by homoacetogenic bacteria, which were also among the closest relatives of the hindgut bacteria, and which recently also proved to have the capability to reduce O_2 with H_2, e.g. *Sporomusa aerivorans* (8,9). Dinitrogen fixation is probably not a major task for symbiotic bacteria in the *Melolontha* hindgut, because the diet these larvae feed on is not as poor in nitrogen than that of wood-feeding termites (C:N ratios in roots of perennial grasses ca. 50 : 1 (36) but up to 1000 : 1 in sound wood (60)). Determination of the sulfate content revealed concentrations of almost 1 mM in the hindgut (30), i.e. that the *Desulfovibrio*-related bacteria could also respire sulfate. The origin of this sulfate, however, is unclear. Ingestion with food (root tissue, soil minerals) appears likely, since sulfate was also detected in the midgut. Plant proteins might be a source of sulfur, which is, however, deliberated during desulfurylation in a reduced form. As no sulfur oxidizing bacteria were detected in the *Melolontha* hindgut, sulfide stemming from S-containing amino acids could be oxidized by the *Desulfovibrio*-related bacteria using molecular oxygen. Finally, sulfate might stem from endogenous sources. Sulfated mucins (sulfomucins; (46)), secreted by gut cells, are a known sulfate source for sulfate reducers in the gastrointestinal tract of higher animals, e.g. of mice (24), however, to our knowledge it is unknown if theses compounds also occur in insect guts. Willis and coworkers (85) could show, that *Bacteroides fragilis* and *Desulfovibrio desulfuri-*

cans could be cocultured with sulfomucin as a single metabolizable substrate. *B. fragilis* released sulfate from sulfomucin using a sulfatase and utilized the remaining mucins as a carbon and energy source. In turn, released fatty acids and sulfate permitted growth of *D. desulfuricans*. Indeed our clonal analyses showed, that *Desulfovibrio*- and *Bacteroides* related species occur together at the hindgut wall.

Archaeal community. Analysis of cloned archaeal 16S rRNA genes corroborated the T-RFLP results presented in the companion study (30). The archaeal hindgut flora of *Melolontha melolontha* larvae is of a very low diversity and consists exclusively of *Methanobrevibacter*-related, probably methanogenic *Euryarchaeota*, closely related to molecular isolates from the hindgut of humivorous *Pachnoda ephippiata* larvae (31) and soil-feeding termites (*Cubitermes orthognathus*) (33). FISH-counts with *Archaea*-specific probes corroborated the absence of any *Archaea* in the midgut compartment, from where no archaeal 16S rRNA genes could be amplified by PCR (30). The absence of *Archaea* from the midgut and the occurrence of clones closely related to methanogenic *Archaea* corroborates the restriction of methanogenesis to the hindgut of *Melolontha* larvae, which was also reported from humivorous *P. ephippiata* and other scarabaeid beetle larvae (38,50). In contrast to other insect gut systems (31,33,75), no 16S rRNA genes of other methanogenic (e.g. *Methanosarcinales*) or non-methanogenic *Euryarchaeota* (e.g. mesophilic *Thermoplasmales*) or *Crenarchaeota* were detected. With 0.2 - 0.3% of all bacterial cells the abundance of archaeal cells in the *Melolontha* hindgut was more comparable to wood-feeding termites (0.1 - 1.8%) than to soil-feeding ones (1.4 - 3.2%) (11) and lower than in humivorous *P. ephippiata* larvae (0.6 - 1.0%; M. Egert, A. Brune and M. W. Friedrich, unpublished results). The apparent lower abundance of *Archaea* in the *Melolontha* gut corroborates the lower methane emission rate compared to *Pachnoda ephippiata* larvae, reported in the companion study (30). These results resemble a trend observed for termites, that soil-feeding species emit more methane and harbor more methanogenic *Archaea* than wood-feeding species (11,12). FISH-counts also revealed a ca. 21x enrichment (based on g fresh weight) of archaeal cells at the hindgut wall compared to the hindgut lumen. A dense population of archaeal cells at the hindgut wall was reported for the wood-feeding termite *Reticulitermes speratus* (49) and helps explain, why the addition of exogenous hydrogen stimulates methanogenesis in excised *Melolontha* hindguts (30). However, compared to other bacteria the fraction of *Archaea* in the biofilm at the hindgut wall is small (1.3%).

Conclusions. The results presented in this study show, that the spatial distribution of microbial communities is needed to gain a deeper understanding of their function in the digestive processes in the intestinal tract of insects. We could show that the microbial community at the hindgut wall of *Melolontha* larvae is considerably different to the community in the hindgut lumen, with the exclusive occurrence of *Desulfovibrio*-related bacteria at the hindgut wall being one of the most interesting findings. To determine, which of the possible functions, outlined in this paper, are actually fulfilled by these bacteria, isolates are needed to determine their physiological capabilities. Furthermore, a detailed analysis of the different sulfur species in the ingested food and the different gut compartments would allow to answer, which sulfur species (if any) are used by the *Desulfovibrio*-like bacteria and where they might come from. Finally, a more detailed analysis of the structure of the biofilm at the hindgut wall is needed to elucidate the relative position of different bacterial groups to each other (e.g. of *Desulfovibrio* and *Bacteroides* species) and to the hindgut wall, from where oxygen penetrates into the gut.

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3.6 Direkte Visualisierung *Desulfovibrio*-verwandter Bakterien an der Enddarmwand von Maikäferlarven

Im Rahmen eines Einführungskurses in die konfokale Laser-Scanning-Mikroskopie (CLSM) unter Leitung von Dr. Thomas Neu (UFZ Leipzig-Halle, Magdeburg) wurden Aufnahmen angefertigt, die die *Desulfovibrio*-verwandten Bakterien im Biofilm an der Maikäfer-Enddarmwand direkt sichtbar machten (Abb. 3.2).

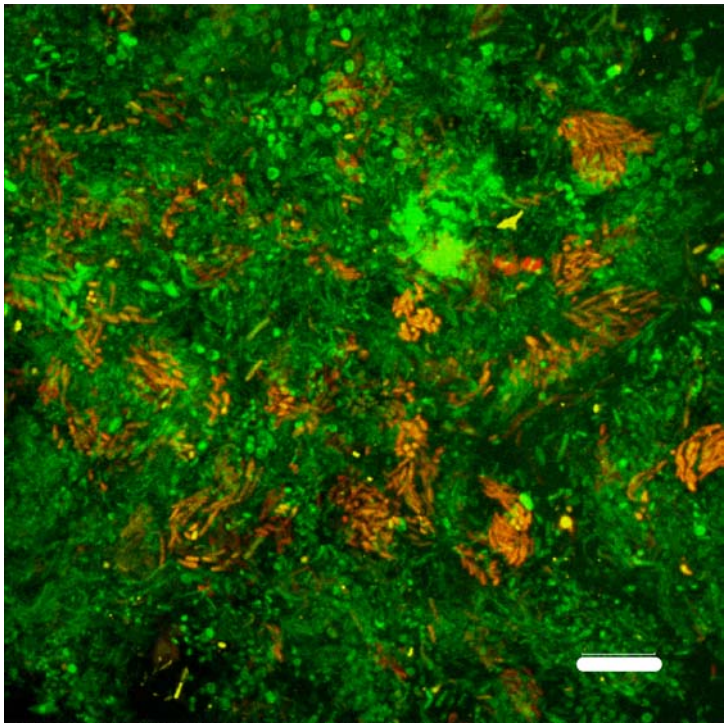

Abbildung 3.2: *Desulfovibrio*-verwandete Bakterien (orange) und andere Bakterien (grün) im Biofilm an der Enddarmwand einer Feldmaikäfer-Larve. Zweidimensionale Projektion von 67 CLSM-Einzelbildern, aufgenommen in 0,3 µm Schritten über eine Tiefe von 19,8 µm. Maßstab: 8 µm. Eingesetzte Sonden: EUBI-Fluorescein und SRB385w-Cy3.

Eine Analyse der 16S rRNA-Gen Klonbibliothek der Maikäfer-Enddarmwand mit der probe-match Funktion der Phylogenie-Software ARB legt nahe, dass durch die eingesetzte SRB385w-Sonde lediglich die *Desulfovibrio*-verwandten Bakterien detektiert wurden. Alle *Desulfovibrio*-Klone wiesen zwar einen randständigen Mismatch am 5´-Ende zur SRB385w-Sonde auf, Zählungen mit der SRB385-Sonde (Kapitel 3.5) zeigten aber, dass die Sequenzen trotzdem detektiert werden. Zahlreiche andere Klone, zumeist Clostridien-Verwandte, hatten zwar auch lediglich einen Mismatch zur SRB385w-Sonde, allerdings in der Mitte der Zielsequenz, wodurch eine Detektion offensichtlich weitgehend verhindert wurde (s. Kapitel 3.5).

Mit Hilfe spezieller Software war es möglich, aus den Abbildung 3.2 zugrunde liegenden 67 Einzelbildern eine dreidimensionale Projektion zu erzeugen, die die Lage der *Desulfovibrio*-Verwandten im bakteriellen Biofilm der Enddarmwand zeigt (Abb. 3.3).

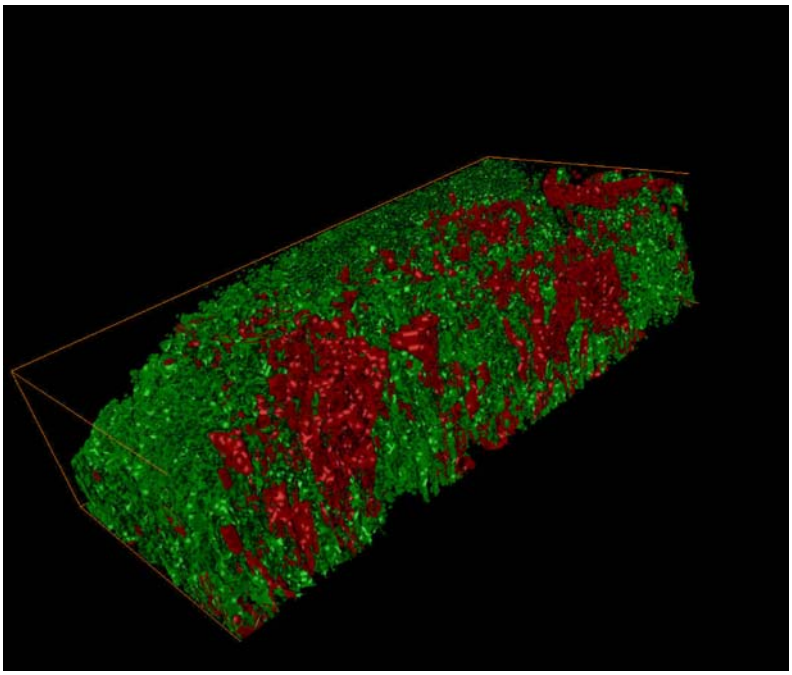

Abbildung 3.3: Dreidimensionale Projektion des Biofilms an der Enddarmwand einer Feldmaikäfer-Larve (rot: *Desulfovibrio*-Verwandete; grün: andere Bakterien), basierend auf 67 CLSM-Einzelbildern (s. Abb. 3.2). Abmessungen der Projektion: ca. 20 x 40 x 80 µm.

Die genaue Orientierung der Projektion ist unklar, tendenziell liegt oberhalb der Projektion das Enddarmlumen und unterhalb die Enddarmwand.

3.7 Analyse mikrobieller 16S rRNA-Genprofile in Boden, Darm und Losung von *Lumbricus terrestris* unter zwei verschiedenen Fütterungsbedingungen

Markus Egert, Sven Marhan, Bianca Wagner, Stefan Scheu und Michael W. Friedrich

Zusammenfassung: In dieser Studie wurden mikrobielle Gemeinschaften (*Bacteria* und *Archaea*) in Futterboden (mit und ohne Zusatz von Buchenstreu), Mitteldarm und Losung von Regenwürmern (*Lumbricus terrestris*, *Lumbricidae, Oligochaeta*) anhand von 16S rRNA-Gen T-RFLP-Profilen miteinander verglichen. Ziel war, den Einfluss der Darmpassage auf die Zusammensetzung der mikrobiellen Gemeinschaft im gefressenen Boden zu verfolgen sowie nach möglichen Hinweisen auf eine spezifische Flora im Regenwurmdarm zu suchen. Ein Vergleich der erzeugten T-RFLP-Profile mit Hilfe ökologischer Diversitäts- und Ähnlichkeitsindizes wies nur marginale Unterschiede zwischen den mikrobiellen Gemeinschaften in Boden, Mitteldarm und Losung unter beiden Fütterungsbedingungen nach, insbesondere im Vergleich zu anderen Boden fressenden Invertebraten. Mit Hilfe einer multivariaten Statistikmethode konnte allerdings gezeigt werden, dass diese Unterschiede sehr signifikant waren, v.a. mit zusätzlichem Buchenstreu als Futter. Aus der großen Ähnlichkeit aller T-RFLP-Profile und dem Fehlen Darm-spezifischer T-RFs wurde gefolgert, dass der Regenwurm vermutlich keine spezifische Darmflora besitzt. Mikroorganismen gelangen v.a. über das Futter in den Darm, wo sich ihre relative Zusammensetzung zwar nur geringfügig, aber doch sehr signifikant verändert.

Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae)

(submitted to FEMS Microbiol. Ecol.)

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Abstract

Earthworms are important members of the soil macrofauna. They modify soil physical properties, soil organic matter decomposition, and thus regulate carbon and nitrogen cycling in soil. However, their interactions with soil microorganisms are still poorly understood, in particular the effect of gut passage on the community structure of ingested microorganisms. Moreover, it is still unsolved, if earthworms, like many other soil-feeding invertebrates, possess an indigenous gut flora. Therefore, we investigated the bacterial and archaeal community structure in soil (with and without additional beech litter), gut and fresh casts of *Lumbricus terrestris*, an anecic litter-feeding earthworm, by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments. Ecological indices of community diversity and similarity, calculated from the T-RFLP profiles, revealed only small differences between the bacterial and archaeal communities in soil, gut and fresh casts under both feeding conditions, especially in comparison to other soil-feeding invertebrates. However, multivariate statistical analysis combining multidimensional scaling and discriminant function analysis proved that these differences were highly significant, in particular when the earthworms were fed beech litter in addition. Because there were no dominant gut-specific OTUs detectable, the existence of an abundant indigenous earthworm flora appears unlikely, at least in the midgut region of *Lumbricus terrestris*.

1. Introduction

At the end of the 19th century Charles Darwin was one of the first who recognized and described the great importance of earthworm activity for the quality of soils [1]. Today it is

widely accepted that earthworms, like other representatives of the soil macrofauna, modify soil physical properties, affect soil organic matter decomposition, and thus regulate carbon and nitrogen cycling in soil [2,3,4]. A major part of the beneficial effects of earthworm activity on soil properties is contributed to interactions with soil microorganisms (for reviews see [5,6]). However, these interactions are still poorly understood, including the effect of gut passage on the community structure of ingested soil microorganisms.

Earthworm guts may be considered as favorable habitats for bacteria, because several studies showed increased microbial numbers in the guts versus the soil, the earthworms were living in [7,8,9,10,11]. In contrast, an increase in microbial biomass by the gut passage was not always observed [12,13,14]. While some early studies proposed that the earthworm gut flora is qualitatively not much different to the microbial community in the surrounding soil [7,8], later studies found significant differences for selected phylogenetic groups or functional guilds of microorganisms, e.g. *Proteobacteria* [15], *Actinobacteria* [16], denitrifiers [17,18] or cellobiose utilizers [19]. Compared to bacteria, only little attention was paid to the archaeal community in earthworm guts or casts [17,18,20]. Up to now only a few studies claimed that earthworms (including *Lumbricus terrestris*) have an indigenous gut flora [21,22], which is typical for other soil-feeding invertebrates, e.g. soil-feeding termites [23,24].

In this study we compared the overall community structure of *Archaea* and *Bacteria* in soil, gut, and fresh casts of *Lumbricus terrestris* by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments. This molecular fingerprinting technique (for reviews see [25,26]) was recently applied to investigate the microbial community structure in soil and gut of soil-feeding termites [27,28] and humivorous beetle larvae [29]. We chose *Lumbricus terrestris*, a large, deep burrowing (anecic sensu Bouché [30]) earthworm as model organism, because this species dominates earthworm biomass in many temperate ecosystems and strongly affects organic matter transformation and soil development [31,32]. We investigated if, and to which extent, the passage through the *Lumbricus* gut actually changes the soil microbial community and if there are indications for a gut-specific flora. Moreover, we tested if addition of beech litter to the soil affects the potential differences in microbial community structure between soil, gut and casts of this earthworm species.

2. Materials and Methods

2.1. Earthworms and feeding conditions

Adult earthworms (*Lumbricus terrestris* L.) were obtained by formalin extraction in an oak-beech forest 20 km south of Darmstadt ("Jägersburger Wald", Germany), washed twice with distilled water and kept at 5°C in containers with soil (see below). One week before the experimental onset the earthworms were shifted to 20°C.

Soil and beech leaf litter were obtained from the "Göttinger Wald" plateau, a submontane beechwood forest on limestone, about 8 km east of Göttingen (Germany), with a canopy layer consisting almost exclusively of 115-120 yr old beech (*Fagus sylvatica*) trees (for further details see [33]). Soil (rendzina) was taken from the top ten centimeters in October 2001, sieved (< 4 mm) and frozen (-28°C) until the experiment was started. Overwintered beech litter was collected from the soil surface at the same time, air-dried and mechanically fragmented to pieces < 4 mm.

The feeding experiment was set up with three independent replicate microcosms per feeding condition, resulting in 3 replicate soil, gut and cast samples per feeding condition, respectively. In detail, the experiment was conducted in two vertically arranged vessels, each consisting of two transparent planar PVC sheets (650 x 310 mm) separated by solid non-transparent PVC strips (10 mm thick) on either side, at the bottom, and a pierced one on the top. Each vessel was separated in three compartments (650 x 100 mm) by further plastic-strips. These six vessel compartments were filled with soil at a level of 500 mm. Soil bulk density was adjusted to 0.70 kg dry weight l^{-1}, moisture content was kept constant at 70% (dry wt). Three randomly chosen vessel compartments were supplemented with 1 g beech litter, placed on top of the soil ([+]-beech litter treatment). Five days before the earthworms were placed in the vessels, they were kept on wet filter paper to get rid off their gut content. One *L. terrestris* specimen (mean body mass 3.2 ± 0.5 g) was put into each compartment and the vessels were subsequently incubated for three days at 20°C in the dark.

2.2 Sampling and DNA extraction

After incubation, all six earthworms were killed, washed, and frozen at -20°C. The litter added on top of the three [+]-beech litter microcosms had totally disappeared during incubation, indicating a consumption by the earthworms. Fresh casts (0-3 days old) were carefully collected in each compartment and also frozen. Soil samples in the [-]-beech litter treatment were taken at least 50 mm away from the nearest burrow, where they have not been affected

by earthworm activity. In the [+]-beech litter treatment, three soil samples carefully mixed with 1 g of beech litter each and incubated for 3 days under the same conditions as the vessels, served as control [12]. Earthworms were dissected with sterile instruments. The gut behind the gizzard was equally divided into three parts, of which the middle one was used for DNA extraction. The used gut section approximately corresponded to the midgut sections A and B as depicted in [34], which were shown to posses many potential attachment sites for microorganisms [21].

DNA was extracted from soil, gut (wall plus content) and cast samples (ca. 0.5 g) following a bead-beating protocol for cell disruption as described by Henckel et al. [35]. The protocol was slightly changed as DNA was purified from the supernatant with phenol/chloroform/isoamylalcohol (25:24:1) instead of ammonium acetate, followed by consecutive isopropanol and ethanol precipitation. Humic substances were removed with polyvinylpolypyrrolidone-filled spin-columns as described previously [29].

2.3. T-RFLP analysis

16S rRNA genes were specifically amplified using the primer combination of 6-carboxyfluorescein (FAM)-labeled primer 27f (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) [36] and 907r (5′-CCG-TCA-ATT-CCT-TTR-AGT-TT-3′) [37] for *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) [38] and FAM-labeled Ar915r (5′-GTG-CTC-CCC-CGC-CAA-TTC-CT-3′) [39] for *Archaea*. The standard reaction mixture contained, in a total volume of 50 µl, 1x PCR buffer II (Applied Biosystems, Weiterstadt, Germany), 1.5 mM $MgCl_2$, 50 µM of each of the four deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 µM of each primer (MWG Biotech, Ebersberg, Germany), 10 ng of bovine serum albumin (Roche, Mannheim, Germany), and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). In addition, 1 µl of a 1:100 dilution of DNA extract (soil, gut or casts) was added as template. All reactions were prepared at 4°C in 0.2 ml reaction tubes to avoid non-specific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94°C) block of a Gene Amp 9700 Thermocycler (Applied Biosystems). The standard thermal profile for the amplification of 16S rRNA genes was as follows: initial denaturation (94°C, 3 min), followed by 32 (*Bacteria*) or 35 (*Archaea*) cycles of denaturation (94°C, 30 s), annealing (52°C, 45 s) and extension (72°C, 90 s). After terminal extension (72°C, 5 min), samples were stored at 4°C until further analysis. Aliquots (5 µl)

of 16S rRNA gene amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide. PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany).

Prior to digestion, amplicon concentrations were determined photometrically. 75 ng of DNA, 2.5 U of restriction enzyme (*Msp*I [bacterial amplicons] or *Alu*I [archaeal amplicons]; Promega, Mannheim, Germany), 1 µl of 10x incubation buffer, and 1 µg of bovine serum albumin were combined in a total volume of 10 µl and digested for 3 h at 37°C. Preliminary tests with *BstU*I and *Hae*III as restriction enzymes, performed with a subset of samples, revealed results highly comparable to those obtained with *Msp*I regarding the similarity of soil, gut and casts samples. To allow for a comparison of the results obtained with *L. terrestris* to results obtained recently for humivorous beetle larvae [29] and soil-feeding termites [28], we chose *Msp*I as restriction enzyme. Fluorescently labeled T-RFs were size separated on an ABI 373A automated sequencer (Applied Biosystems) using an internal size standard (GeneScan-1000 ROX; Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems).

2.4. Statistical analysis

Prior to statistical analysis all bacterial and archaeal T-RFLP electropherograms (starting from 50 bp to exclude T-RFs caused by primer dimers) were normalized to identical total peak heights, respectively, using an iterative method described by Dunbar et al. [40], where all T-RFs with a height less than 50 relative fluorescence units were omitted. All subsequent calculations were performed with major T-RFs, arbitrarily defined as those with a relative peak height of ≥ 1.5% of the total electropherogram peak height. To ensure that the relative heights of all major T-RFs equal 100%, they were normalized to the total peak height of all major T-RFs per electropherogram. As the size of identical T-RFs can vary in a range of 1-2 bp among different gels and/or lanes of the same gel, major T-RFs similar in size of ± 1-2 bp were summarized to operational taxonomic units (OTUs). Applying the above-mentioned 1.5% threshold for the definition of major T-RFs, ca. 30 different bacterial and archaeal OTUs each could be created from the analyzed T-RFLP profiles, which were used for the statistical analysis of the different samples.

For the calculation of ecological indices, OTUs were treated as species and their relative height served as a measure of relative abundance. Shannon-Wiener indices and (Shannon-)

evenness [41] were used to compare diversity among the different samples and treatments. Evenness values were calculated using the natural logarithm of the number of OTUs per single electropherogram. To describe the pair wise similarity of microbial communities, e.g. between soil and gut, Morisita indices of community similarity were used as described in [42]. Morisita indices range from 0 to 1, with 1 indicating complete (100%) identity of two communities. Differences in ecological indices between different samples and treatments were checked for statistical significance ($P < 0.05$) using non-parametric tests (Mann-Whitney, Kruskal-Wallis).

To compare the overall structure of bacterial and archaeal communities among the different compartments (soil, gut, casts) and treatments (with and without beech litter) multidimensional scaling (MDS) and discriminant function analysis (DFA) were used, based on a scheme proposed by Puzachenko and Kuznetsov [43], previously applied for microfungal communities by Tiunov and Scheu [44]. In detail, a square matrix of nonparametric Gamma correlation (analogous to Kendall τ) was calculated from the relative frequencies of all bacterial and archaeal OTUs, respectively. This matrix was analyzed by multidimensional scaling, i.e. an ordination technique, which "rearranges" objects in a max. nine-dimensional space, so as to arrive at a configuration that best approximates the observed distances. The number of meaningful dimensions was evaluated by comparing actual stress values with the theoretical exponential function of stress. The coordinates of the samples in the *n*-dimensional space were used for discriminant function analysis, with "compartment" (soil, gut, casts) as a grouping variable. Squared Mahalanobis distances between group centroids and reliability of sample classification were determined. Typically, only two significant discriminatory roots were derived and, therefore, the results of DFA were graphically presented in two dimensions. For the interpretation of the discriminant axes with respect to the frequency of OTUs, linear correlations were calculated between the discriminant function scores for each sample and the relative OTU frequencies. All MDS and DFA calculations were performed using the STATISTICA (6.0) software package.

3. Results

Archaeal and bacterial 16S rRNA gene fragments were successfully amplified from six soil, six gut and six cast DNA extracts, respectively (three per feeding condition, each). All results

are based on the analysis of normalized 16S rRNA gene fingerprints obtained from three independent replicate samples per compartment and feeding condition, respectively.

3.1. Archaeal community structure

The first section addresses the influence of gut passage on the archaeal community structure, which, in contrast to the bacterial community, has so far been scarcely investigated. The T-RFLP profiles of the archaeal community structure in soil, gut and fresh casts of *L. terrestris* were similar, in particular without beech litter as additional food source for the earthworms (Fig. 1A). None of the calculated ecological indices (Table 1) revealed significant differences in the community structure among soil, gut or casts under both feeding conditions.

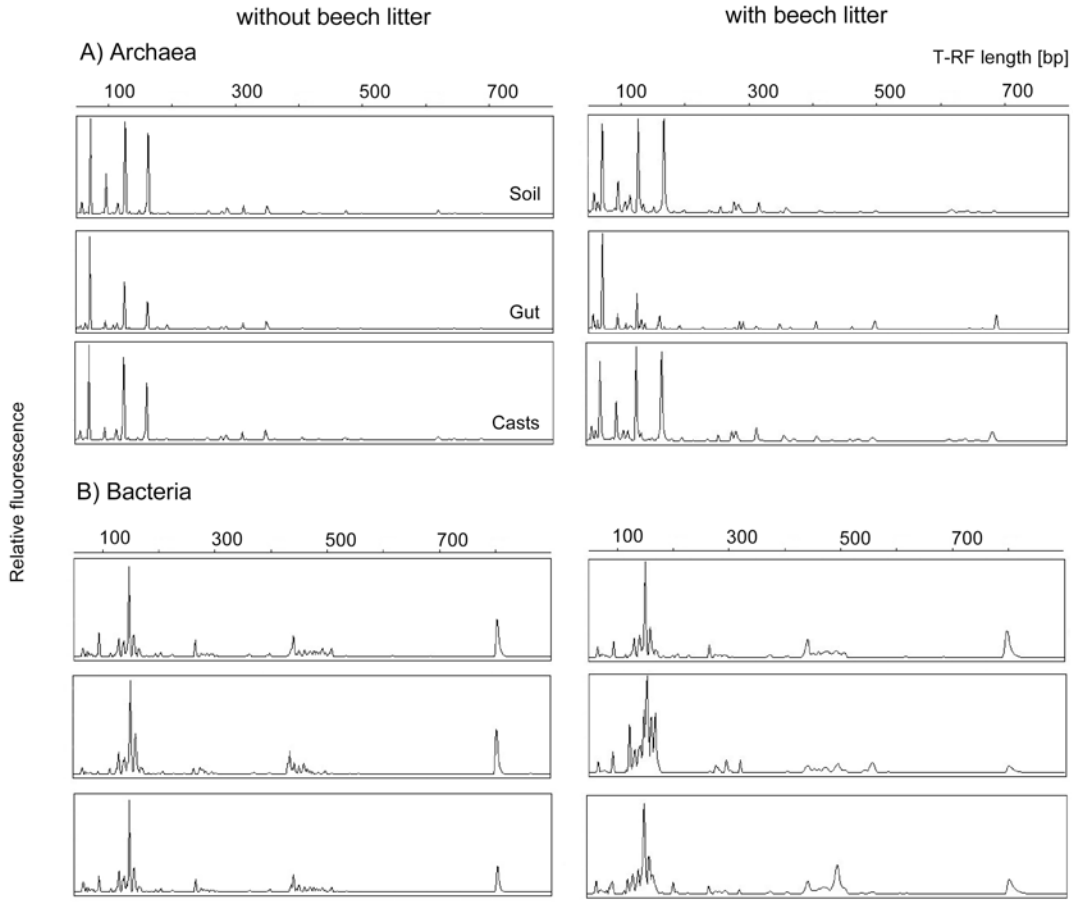

Figure 1. Exemplary T-RFLP profiles of archaeal (A) and bacterial (B) 16S rRNA genes amplified from DNA extracts of soil, gut, and fresh casts of two *Lumbricus terrestris* earthworms. One was kept for three days without litter (left side), the other one with additional beech litter (right side) in its soil, respectively. *Alu*I (*Archaea*) and *Msp*I (*Bacteria*) were used for restriction digest. Note that in total three soil, gut, and cast samples each were investigated per feeding condition, resulting in 18 archaeal and 18 bacterial fingerprints to be compared (of which only 6 each are depicted here).

Table 1: Diversity and similarity indices characterizing the archaeal community structure in soil, gut, and fresh casts of *Lumbricus terrestris* under two feeding conditions (FC; with [+] and without [–] additional beech litter), based on T-RFLP analysis of 16S rRNA gene fragments. Values are mean ± SEM (n = 3, for each compartment and treatment). Means within rows and columns followed by the same letter are not significantly ($P < 0.05$) different.

Index	*FC*	**Soil**	**Gut**	**Casts**
Diversity (Shannon-Wiener)	−	1.68 ± 0.21 a	1.89 ± 0.17 a	1.61 ± 0.19 a
	+	2.12 ± 0.05 b	2.03 ± 0.15 ab	2.21 ± 0.06 b
Evenness	−	0.76 ± 0.02 a	0.77 ± 0.04 a	0.73 ± 0.04 a
	+	0.82 ± 0.01 b	0.79 ± 0.02 ab	0.83 ± 0.01 b
		Soil : Gut	**Soil : Casts**	**Gut : Casts**
Similarity (Morisita)	−	0.92 ± 0.02 a	0.96 ± 0.01 a	0.93 ± 0.01 a
	+	0.77 ± 0.08 a	0.98 ± 0.01 a	0.80 ± 0.05 a

However, the addition of beech litter to the soil had a significant influence, when corresponding compartments were compared. Based on Shannon-Wiener and evenness values, litter significantly ($P < 0.05$) increased the archaeal diversity in soil and casts, but not in the gut. In contrast, litter did not affect community similarity. Morisita indices in the range of 0.92 – 0.96 indicated that the archaeal community was almost identical in soil, gut and casts, when no litter was added. Addition of litter lowered the average similarity of the archaeal community in the guts to the communities in soil and casts, however not statistical significantly.

In contrast to the ecological indices, the combination of multidimensional scaling and discriminant function analysis detected significant differences between the investigated compartments (soil, gut, casts) under both feeding conditions. Multivariate statistical analysis of all archaeal OTU frequencies resulted in two discriminant axes, depicted in Fig. 2.

OTUs showing a significant correlation to one of these axes, i.e. OTUs responsible for the separation of the compartments, are given in Table 2. Without beech litter, T-RFLP profiles of earthworm guts were significantly separated from the soil profiles, but not from the cast profiles (Fig. 2; for squared Mahalanobis distances and levels of significance for discrimination of the different compartments see Table 3).

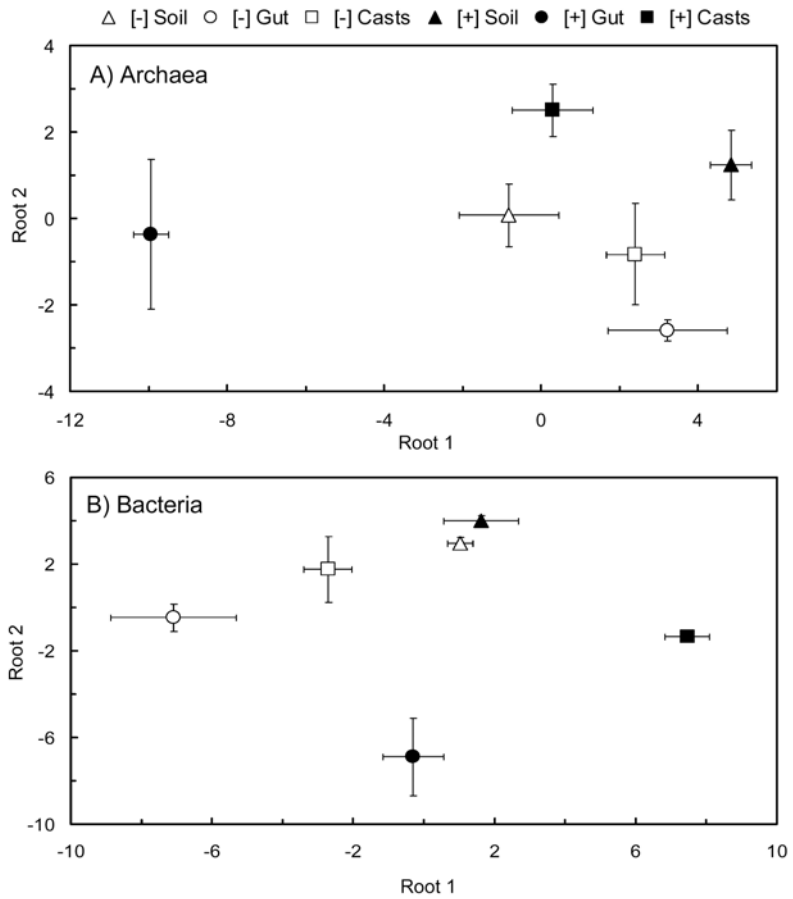

Figure 2. Discriminant function analysis of the relative frequencies of archaeal (A) and bacterial (B) OTUs in T-RFLP profiles of 16S rRNA genes amplified from soil, gut and fresh casts of *Lumbricus terrestris* earthworms kept for three days with ([+], closed symbols) and without ([-], open symbols) additional beech litter in their soil, respectively. Shown are group centroids of treatments with standard deviations for root 1 and root 2. Each centroid is representing three independent replicates. In case of the archaeal OTUs, root 1 represents 87.2% ($P < 0.0001$) of the variance, root 2 9.6% ($P < 0.05$). In case of the bacterial OTUs root 1 represents 56.5% ($P < 0.0001$) of the variance, root 2 37.4% ($P < 0.0001$).

There was also no significant discrimination between cast and soil profiles. The addition of litter to the soil markedly increased the differences in the archaeal community structure. Under this feeding condition, all investigated compartments were significantly discriminated from each other. Comparing corresponding compartments, addition of litter significantly changed the archaeal community structure in soil and guts, but not in the casts of the earthworms. When the statistical analysis was expanded from major T-RFs (with a relative peak height of ≥1.5% of the total electropherogram peak height) to all T-RFs in the normalized electropherograms, casts were discriminated from the guts even without additional beech litter, however, casts with and without litter remained indiscriminate (data not shown).

Table 2: Relative percentile frequencies (mean ± SEM; n = 3, for each compartment and treatment) of archaeal OTUs (bp) with a linear correlation (*r*-values) to one of the two discriminant axes (see Fig. 2). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. n.d. = OTU not detected.

	without additional litter			with additional litter			correlation	
OTU	**Soil**	**Gut**	**Casts**	**Soil**	**Gut**	**Casts**	**Axis 1**	**Axis 2**
108	n.d.	2.8 ± 0.8	0.5 ± 0.5	3.9 ± 0.6	0.7 ± 0.7	3.0 ± 0.2	–0.449	–0.650**
114	3.0 ± 1.8	3.5 ± 0.5	2.8 ± 0.5	5.2 ± 1.5	n.d.	3.3 ± 0.6	–0.552*	–0.136
126	32.6 ± 4.6	22.7 ± 1.2	34.3 ± 4.9	21.6 ± 1.1	13.0 ± 0.5	22.0 ± 0.4	–0.492*	0.221
274	n.d.	n.d.	n.d.	2.7 ± 0.2	n.d.	2.4 ± 0.1	–0.245	–0.742***
286	1.2 ± 0.6	n.d.	0.6 ± 0.6	1.9 ± 0.1	3.9 ± 1.4	2.9 ± 0.2	0.705**	–0.330
409	n.d.	n.d.	n.d.	n.d.	3.1 ± 0.7	n.d.	0.848***	0.005
498	n.d.	n.d.	n.d.	n.d.	2.9 ± 1.5	n.d	0.708**	–0.119
687	n.d.	n.d.	n.d.	n.d.	5.5 ± 0.9	2.5 ± 0.2	0.832***	–0.196
800	n.d.	n.d.	n.d.	n.d.	0.8 ± 0.8	n.d	0.638**	–0.146

To find possible indications for an indigenous earthworm flora, archaeal T-RFLP-profiles were screened for gut-specific OTUs, defined as occurring in the majority (at least 4 out of 6) of earthworms, i.e. under both feeding conditions, exclusively in the gut sections or at least in gut and cast profiles, but not in the soil profiles. However, not a single archaeal OTU (out of 29) fulfilled these requirements.

3.2. Bacterial community structure

Like the archaeal profiles, the bacterial profiles for all compartments were similar, particularly without additional litter (Fig. 1B). Evenness values and Morisita indices of community similarity were not significantly different, when soil, guts and casts were compared for both feeding conditions (Table. 4). With additional litter, OTU diversity in the casts, based on Shannon-Wiener indices, was significantly ($P < 0.05$) higher compared to gut and soil. When corresponding compartments were compared, the addition of beech litter had a significant

Table 3: Squared Mahalanobis distances between group centroids and reliability of discrimination based on relative frequencies of archaeal and bacterial (in italics) OTUs. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; [-] = without additional beech litter; [+] = with additional beech litter.

	[-] Soil	**[-] Gut**	**[-] Casts**	**[+] Soil**	**[+] Gut**	**[+] Casts**
[-] Soil	0	44.8*	21.2	58.8**	134.0***	19.0
[-] Gut	*124.5****	0	7.4	26.2	267.3***	52.0**
[-] Casts	*28.3**	*43.7**	0	16.3	229.7***	24.6
[+] Soil	*19.7*	*152.6****	*50.4***	0	331.3***	33.6*
[+] Gut	*153.6****	*137.0****	*126.0****	*187.8****	0	169.5***
[+] Casts	*97.1***	*319.8****	*174.2****	*106.7****	*143.1****	0

Table 4: Diversity and similarity indices characterizing the bacterial community in soil, gut, and fresh casts of *Lumbricus terrestris* under two feeding conditions (FC; with [+] and without [–] additional beech litter), based on T-RFLP analysis of 16S rRNA gene fragments. Values are mean ± SEM (n = 3, for each compartment and treatment). Means within rows and columns followed by the same letter are not significantly ($P < 0.05$) different.

Index	*FC*	**Soil**	**Gut**	**Casts**
Diversity (Shannon-Wiener)	−	2.13 ± 0.09 a	2.11 ± 0.05 a	2.17 ± 0.03 a
	+	2.37 ± 0.02 b	2.32 ± 0.01 b	2.51 ± 0.02 c
Evenness	−	0.89 ± 0.01 a	0.86 ± 0.01 a	0.88 ± 0.02 a
	+	0.86 ± 0.01 b	0.89 ± 0.01 ab	0.90 ± 0.01 ab
	FC	**Soil : Gut**	**Soil : Casts**	**Gut : Casts**
Similarity (Morisita)	−	0.94 ± 0.01 a	0.96 ± 0.02 a	0.94 ± 0.01 a
	+	0.77 ± 0.07 b	0.91 ± 0.01 ab	0.89 ± 0.03 ab

influence, in particular on the diversity indices. Shannon-Wiener indices increased significantly ($P < 0.05$) in all compartments, when litter was added to the soil. The evenness decreased in the soil, while it remained unaffected in guts and casts. Community similarity be-

tween soil and guts (94%), determined by means of Morisita indices, significantly ($P < 0.05$) decreased when the earthworms ingested additional litter.

Table 5: Relative percentile frequencies (mean ± SEM; n = 3, for each compartment and treatment) of bacterial OTUs (bp) with a linear correlation (*r*-values) to one of the two discriminant axes (Fig. 2). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. n.d. = OTU not detected.

	without additional litter			with additional litter			correlation	
OTU	**Soil**	**Gut**	**Casts**	**Soil**	**Gut**	**Casts**	**Axis 1**	**Axis 2**
91	6.5 ± 0.4	n.d.	3.7 ± 0.7	5.0 ± 0.3	2.1 ± 1.2	4.6 ± 1.1	0.636**	0.469*
121	n.d.	n.d.	n.d.	n.d.	7.2 ± 1.4	3.8 ± 0.1	0.347	–0.895***
147	5.1 ± 0.4	5.3 ± 1.1	5.1 ± 1.3	3.0 ± 0.1	11.5 ± 2.0	6.4 ± 0.8	0.012	–0.904***
153	27.3 ± 0.7	26.9 ± 0.8	27.8 ± 0.5	31.3 ± 0.8	20.6 ± 2.6	25.4 ± 1.3	–0.049	0.860***
160	13.2 ± 0.9	19.9 ± 1.3	14.8 ± 0.8	10.0 ± 0.1	11.9 ± 1.2	8.9 ± 0.3	–0.855***	0.066
165	n.d.	n.d.	n.d.	0.9 ± 0.9	13.8 ± 3.3	10.0 ± 0.6	0.462	–0.848***
199	n.d.	n.d.	n.d.	n.d	n.d	4.8 ± 0.9	0.722**	–0.157
266	4.8 ± 0.2	n.d.	3.2 ± 0.6	4.0 ± 0.1	n.d.	2.3 ± 0.2	0.393	0.770***
295	n.d.	n.d.	n.d.	n.d.	2.0 ± 1.1	n.d.	–0.015	–0.751***
439	7.0 ± 0.8	9.0 ± 1.3	6.4 ± 0.7	5.9 ± 0.3	1.7 ± 0.9	3.4 ± 0.2	–0.594**	0.608**
450	n.d.	1.8 ± 0.9	0.7 ± 0.7	0.6 ± 0.6	0.6 ± 0.6	n.d	–0.583*	–0.090
471	n.d	2.9 ± 0.1	0.9 ± 0.9	2.0 ± 0.1	n.d	0.6 ± 0.6	–0.467*	0.288
488	n.d	n.d	n.d	n.d	n.d	2.1 ± 1.1	0.604**	–0.127
496	1.7 ± 0.9	n.d	n.d	2.2 ± 0.0	2.4 ± 0.5	5.7 ± 1.0	0.840***	–0.247
557	n.d	n.d	n.d	n.d	2.3 ± 1.4	n.d	–0.027	–0.712**
798	15.6 ± 2.9	11.9 ± 1.1	11.3 ± 4.4	7.7 ± 0.8	1.6 ± 0.9	4.0 ± 0.3	–0.353	0.617**

While the ecological indices revealed only little differences between the investigated compartments, the multivariate statistics approach again was highly effective in discrimination. Statistical analysis of the frequencies of all bacterial OTUs resulted in five discriminant axes, two of which are depicted in Fig. 2. OTUs with a significant correlation to one of these two axes, i.e. OTUs that are responsible for the separation of the compartments, are given in Table 5. Under both feeding conditions earthworm guts, casts and soil were significantly discriminated. However, the discrimination was more pronounced, when litter was added (Fig. 2; for squared Mahalanobis distances and levels of significance for discrimination of the different compartments see Table 3). Beech litter significantly changed the bacterial community structure in the gut and cast compartments, but not in the soil. Because we did not aim to investigate the effect of litter addition on the soil microbial community structure, it was not attempted to discriminate the soil samples by lowering the threshold level for major T-RFs.

Also the bacterial T-RFLP-profiles were screened for gut specific OTUs. However, as in case of the archaea, none out of 27 OTUs occurred exclusively in the majority of earthworm guts or casts but not in the soil samples.

4. Discussion

This is the first study, which analyzed the overall bacterial and archaeal community structure in soil, gut and fresh (0-3 days old) casts of an earthworm (*Lumbricus terrestris*) under different feeding conditions using a molecular, culture-independent fingerprinting technique. We investigated the influence of passage through an earthworm gut on the soil microbial community with and without additional beech litter in the soil and sought possible indications for an indigenous (gut-specific) earthworm flora.

4.1. Methodological considerations regarding T-RFLP analysis

T-RFLP analysis is a PCR-based method, which can be biased (e.g., [45,46] for a review see [47]). Therefore, the amplicon pools, obtained by PCR from the different samples in this study, did not necessarily reflect the quantitative composition of the underlying bacterial and archaeal communities. However, studies exists, which showed that PCR-T-RFLP may adequately reflect the relative composition of functional [48] or 16S rRNA genes [49] in model communities. Furthermore, regarding soil type and earthworm species the samples investi-

gated in this study were quite similar, as indicated by the highly similar profiles, and they were all investigated using the same PCR-conditions. Thus, all samples were probably affected equally by a potential PCR-bias, i.e. the comparison of the different fingerprints is probably not affected by this kind of bias. The resolution limit of T-RFLP analysis is another important point to mention. The different OTUs, which are the basis for the comparison of the investigated compartments, do most likely not represent different microbial species but heterogeneous groups of species sharing the same restriction sites, particularly when domain-specific primer systems are used (see [29] for a recent example, where T-RFs are assigned to different phylogenetic groups). However, considering not only the presence or absence of OTUs but also their relative frequency, should provide a good basis for comparing microbial community fingerprints. In other words, if two fingerprints match in presence and frequency of different OTUs, this is indicative of a high similarity of the underlying amplicon pools. A highly comparable PCR-T-RFLP approach as used in this study, using the Morisita index as a measure of fingerprint similarity [42], has just recently proven to be suitable to reveal differences in the microbial community structure between ingested soil and the different gut sections of humivorous beetle larvae [29] and soil-feeding termites [28]. For instance, despite the limited resolution of T-RFLP analysis several bacterial and archaeal T-RFs were found to occur exclusively in the gut of humivorous beetle larvae but not in the soil profiles, indicating indigenous populations of microorganisms, e.g. of gut-specific lactic acid bacteria (see Fig. 4 in [29]).

4.2. Comparison of Lumbricus terrestris *to other soil-feeders*

Based on the calculated Morisita community similarity indices, which compare two communities not only by the presence or absence of species (here: OTUs) but also on their relative abundance [42], the archaeal and bacterial community of the earthworm gut was very similar to the communities in soil and casts, in particular without additional litter as food source for the earthworm (92 – 96% similarity). The addition of beech litter decreased the similarity values, particularly between gut and soil to 77% similarity. However, only in case of the bacterial community this decrease was statistically significant. Compared to other soil-feeding invertebrates (investigated almost identically), the intestinal community of *L. terrestris* was under both feeding conditions very similar to the community in the ingested soil. Egert et al. [29] determined Morisita similarity values of 28 – 33% when comparing the bacterial com-

munity in soil and the two major gut sections of humivorous *Pachnoda ephippiata* larvae (*Coleoptera, Scarabaeidae*), Schmitt-Wagner et al. [28] found similarities ranging from 9 – 27% for the comparison of the bacterial community structure in five different gut sections of the soil-feeding termite *Cubitermes orthognathus* to the soil community. In case of the archaeal community structure, the Morisita similarity between soil and *P. ephippiata* gut sections was 61 – 69% [29], which is also lower than the values determined for *L. terrestris*, in particular without litter as additional food source. The high similarity of the microbial community in the earthworm gut in relation to soil and casts, revealed by the Morisita indices, is corroborated by the determined diversity indices. With one exception, there were no significant differences detectable in the archaeal or bacterial diversity parameters (Shannon-Wiener index, evenness) between soil, gut and casts for each feeding condition. Based on Shannon-Wiener indices, the bacterial OTU diversity in the casts was significantly higher than in soil or gut, when the earthworms were fed litter in addition.

Our findings corroborate the results of other studies that most (if not all) bacteria in the earthworm gut originate from the ingested soil, and are suitable to extend this hypothesis also onto the archaeal community in the earthworm gut. Already in 1913 Bassalik [7] isolated 50 bacterial species from the intestine of *L. terrestris* and found none that was different to bacteria isolated from the soil. Later Parle [8] and Satchell [50] came to the conclusion that there are no indications for a specialized, indigenous earthworm flora which differs greatly from that in the soil. Also recent studies, which applied culture-independent molecular techniques as well as classical isolation-techniques, agree with this hypothesis. Furlong et al. [20] compared the microbial community structure in casts and soil of *Lumbricus rubellus* and conclude that most of the prokaryotic community in the casts is probably derived from the soil. Recently Horn et al. [34] showed that the emission of N_2O by *Aporrectodea caliginosa* is mostly caused by denitrifiers and nitrate-dissimilating bacteria ingested with the soil and activated under the gut conditions and does not originate from an autochthonous, gut-wall associated population of microorganisms.

So far, the only indication for an indigenous gut flora in a *Lumbricus* earthworm species comes from an electron microscopy study. Jolly et al. [21] reported on rod-shaped cells attached to the hindgut wall by “socket-like” structures in 4 out of 10 examined *L. terrestris* species. However, they could not further confirm these results by transmission electron microscopy. We assumed that a dominant indigenous earthworm flora could result in gut-

specific or (allowing some wash-out) at least gut- and cast-specific OTUs that do not appear in the soil profiles. With the same PCR-T-RFLP approach such OTUs could be easily identified in soil-feeding termites [28] and humivorous beetle-larvae [29]. Furthermore, we assumed that these gut-specific OTUs should be more or less independent of the food, consumed by the earthworm. However, none of ca. 30 investigated archaeal and bacterial OTUs each fulfilled these assumptions. Some OTUs were gut-specific or gut- and cast-specific for one feeding condition, but none occurred consistently in the majority of earthworm, i.e. under both feeding conditions, exclusively in the gut sections but not in the soil. These results, together with the high similarity values obtained for the comparison of gut and soil profiles, argue against an abundant indigenous bacterial or archaeal earthworm flora, at least in the midgut section of *L. terrestris*. However, due to the limited resolution of T-RFLP analysis it can not be fully excluded that gut-specific groups of microorganisms could not be differentiated from soil-derived microorganism with the same restriction sites and/or that were discriminated by the used general primer systems.

It is tempting to speculate for some reasons, why the microbial community structure in the earthworm gut is so similar to the soil community, particularly compared to other soil-feeding invertebrates. Humivorous scarab larvae and soil-feeding termites possess a highly compartmentalized gut with two and five distinct fermentation chambers, respectively, in which the physicochemical conditions are greatly different [51,52,53]. The earthworm gut rather resembles a long tube without any distinct chambers [31]. Though a recent study proved considerable differences in some physicochemical parameters between soil and earthworm gut [34], still little is known about physicochemical gradients along the gut axis. They might not be as extreme as in soil-feeding termites or beetle larvae, e.g. regarding intestinal pH values. The earthworm gut undoubtedly possesses some morphological features allowing a differentiation into a fore-, mid- and hindgut, and the typhlosole fold to increase the gut surface [21,31], however, special attachment structures for microorganisms are lacking. In contrast, the hindgut of humivorous cetoniid beetle larvae contains a great number of featherlike chitin-structures, densely covered with a biofilm of microorganisms, including methanogenic archaea [54]. In soil-feeding termites, cuticular spines in the posterior hindgut may have a comparable function [55,56]. These structures might be one reason why the gut flora of humivorous beetle larvae is considerably different from the soil community despite the fact that they

exchange their gut content ca. twice a day [53], which is in the range of earthworms such as *L. terrestris* [8].

4.3. Multivariate statistical analysis of microbial community structures

Several studies encountered significant differences between soil and earthworm gut or casts, when particular groups of microorganisms were focused on (for a review of older studies see [5,31]). For example, using fluorescence in-situ hybridization Schönholzer et al. [15] found a significant reduction of α-, β-, and γ-*Proteobacteria* in the gut of *L. terrestris* compared to the soil, while δ-*Proteobacteria* and members of the CFB-phylum increased in the casts. Using a 16S rRNA gene approach, Furlong et al. [20] detected increased frequencies of *Actinobacteria*-, *Firmicutes*- and *γ-Proteobacteria*-related clones in a cast clone library. To investigate potential differences between soil, earthworm gut and casts in more detail, we also applied a multivariate statistics approach, thereby accounting for the fact that analysis of T-RFLP profiles in our case means comparing ca. 30 different OTUs simultaneously under the influence of different parameters (here: three different compartments under two different feeding conditions).

Combined multidimensional scaling and discriminant function analysis was highly effective in discriminating the different compartments under both feeding conditions. Even without litter as additional food source, the bacterial community in earthworm guts was significantly discriminated from the communities in soil and casts, which also were significantly different from each other. The addition of litter markedly sharpened these differences, as can be deduced from the increased squared Mahalanobis distances (Table 3). In principal, the archaeal community structure was affected in the same way. However, while with litter as additional food source the community in soil, gut and casts was significantly discriminated, without litter only the gut and soil compartment were significantly separated from each other. These significant differences are not necessarily inconsistent with the results obtained with the ecological indices, which indicated a great similarity of all compartments under both feeding conditions, particularly when compared to other soil-feeding invertebrates. The differences in the microbial community structure between earthworm gut, soil and casts are obviously just beyond the resolution limit of the used ecological indices. In other words, the differences in the overall community structures were rather small, but consistent among the replicate samples, and therefore significant, which became obvious, when analyses were performed with more sophisticated methods.

Several potential reasons for differences between the microbial community structure in soil, earthworm gut and casts have been discussed: A) selective feeding of the earthworm on hot spots of microbial life [11]; B) a shift from more oxic conditions in the soil to anoxic conditions in the gut lumen [34] favoring microorganisms able to grow anaerobically [19]; C) proliferation of certain groups of microorganisms, e.g. pseudomonads [20], due to their capability to effectively exploit favorable physicochemical conditions in the earthworm gut (e.g. high contents of organic carbon and nitrogen) [34]; D) differential lysis of certain microbes by digestive enzymes secreted by the earthworm [6]; E) inhibition of certain groups of bacteria caused by inhibitory substances (e.g. antibiotics) secreted by other bacteria, such as a *Actinobacteria* [20,57]. Addition of beech litter to the soil markedly increased the differences between the investigated compartments for both bacteria and archaea. It is most likely that this increase in difference was due to preferential feeding of the earthworms on the supplemented litter (and the microorganisms on it), because *L. terrestris* is a litter-feeding earthworm species, preferentially consuming litter in a mixture with mineral soil [58]. Ingested litter might enhance some of the above-mentioned processes with influence on the microbial community structure, because ingested litter means an additional input of nutrients into the gut that might stimulate the secretion of digestive enzymes by the earthworm. For endogeic *Hormogaster elisae* earthworms it was suggested that at least the amount of mucus added to the gut increases with the content of organic matter in the soil [59]. Parle [8] showed that pure soil passes the gut of *L. terrestris* within about 11-12 h, e.g. when burrows are being formed. However, the time span of gut passage was prolonged to about 20 h, when the animals fed on additionally provided organic matter mixed with soil. This means, additional litter could have increased the effect of gut passage also by prolonging the time the microbial community was exposed to the gut conditions.

4.4. Conclusions, hypotheses, and outlook

Our results show that the bacterial and archaeal community structure in the midgut of *L. terrestris* is significantly different from the communities in the ingested soil and the defecated casts, however, at a level beyond the resolution of conventional ecological indices. The applied multivariate statistics approach combining MDS and DFA was highly effective in analyzing T-RFLP data and appears to be a promising tool for further fingerprint studies, for which appropriate methods of analysis are currently discussed in the scientific community [60]. The microbial community in the earthworm gut seems to be largely soil- or food-

derived, and the significant differences between earthworm midgut and soil or casts probably result from selective feeding and the physicochemical gut conditions, which induce changes in the composition of the ingested microorganisms, and not from a truly indigenous gut flora, for which we could not find any indications. This hypothesis is corroborated by the finding that additional beech litter, preferably consumed by the earthworms, sharply increased the differences between soil, gut, and casts. In comparison to other soil-feeding macroinvertebrates, the bacterial and archaeal community in the intestine of *L. terrestris* is very similar to the community in the ingested soil. The relationship between earthworms and their intestinal microbiota seems to be different than that of soil-feeders with a more pronounced gut-specific flora (which is, however, also far from being fully understood [23,56]). According to Edwards and Fletcher [6], a great similarity between soil and gut flora is indicative that intestinal bacteria (and archaea) are rather food for earthworms than helpers that aid digestion. However, the same authors also conclude that in comparison to fungi and protozoa, soil bacteria are only of little importance as food source.

Future studies could include fingerprinting analysis of 16S rRNA in addition to 16S rRNA genes to directly compare and link changes in microbial activity to changes in microbial community structure. In combination with 16S rRNA gene cloning and sequencing, assignment of OTUs to distinct groups of microorganisms would allow elucidating which soil microorganisms particularly flourish in the earthworm gut and if they could be beneficial for the digestion of *L. terrestris* or earthworm species from other feeding guilds.

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3.8 Pseudo-T-RFs: PCR-Artefakte mit Auswirkungen auf die T-RFLP Analyse mikrobieller Gemeinschaften

Markus Egert und Michael W. Friedrich

Zusammenfassung: In dieser Studie konnten wir zeigen, dass bei T-RFLP-Analysen klonaler Amplikons neben den erwarteten terminalen Restriktionsfragmenten (T-RFs) sehr häufig und reproduzierbar zusätzliche Restriktionsfragmente im T-RFLP-Profil auftreten. Diese zusätzlichen T-RFs, die nach Sequenzanalyse nicht der terminalen sondern einer höheren (z.B. der zweiten und/oder dritten) Restriktionsschnittstelle entsprechen, wurden pseudo-T-RFs genannt, weil sie ohne Kenntnis der zugrunde liegenden Sequenzen in einem T-RFLP-Profil leicht mit „echten“ T-RFs verwechselt werden können. Pseudo-T-RFs konnten auch in Profilen von Reinkulturen und Umweltproben nachgewiesen werden. Eine Behandlung des Amplikonpools mit DNA-Einzelstrang-abbauender Mung bean-Nuklease vor der T-RFLP-Analyse führte zu einem Verschwinden der pseudo-T-RFs. Dies zeigte, dass (partiell) einzelsträngige Amplikons die Ursache für die Entstehung von pseudo-T-RFs sind, weil einzelsträngige DNA von Restriktionsendonukleasen nicht geschnitten wird. Eine starke Abhängigkeit der pseudo-T-RF-Bildung von der PCR-Zyklenzahl deutete an, dass partiell einzelsträngige Amplikons während der PCR entstehen. Wir vermuten, dass sich in den einzelsträngigen Abschnitten mancher Amplikons durch Selbstfaltung Sekundärstrukturen so ausbilden können, dass die terminalen Schnittstellen für Restriktionsenzyme doch zugänglich werden. Um zu verhindern, dass durch das Auftreten von pseudo-T-RFs die mikrobielle Diversität in einer Probe überschätzt wird, wird empfohlen, pseudo-T-RFs über Mung bean-Nuklease-Behandlung zu identifizieren sowie Klonbibliotheken von 16S rRNA-Genen parallel zur T-RFLP-Analyse anzulegen, deren Klone einzeln auf ihr in-vitro T-RF-Bildungsverhalten zu testen sind.

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Formation of Pseudo-Terminal Restriction Fragments, a PCR-Related Bias Affecting Terminal Restriction Fragment Length Polymorphism Analysis of Microbial Community Structure

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Terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified genes is a widely used fingerprinting technique in molecular microbial ecology. In this study, we show that besides expected terminal restriction fragments (T-RFs), additional secondary T-RFs occur in T-RFLP analysis of amplicons from cloned 16S rRNA genes at high frequency. A total of 50% of 109 bacterial and 78% of 68 archaeal clones from the guts of cetoniid beetle larvae, using *Msp*I and *Alu*I as restriction enzymes, respectively, were affected by the presence of these additional T-RFs. These peaks were called "pseudo-T-RFs" since they can be detected as terminal fluorescently labeled fragments in T-RFLP analysis but do not represent the primary terminal restriction site as indicated by sequence data analysis. Pseudo-T-RFs were also identified in T-RFLP profiles of pure culture and environmental DNA extracts. Digestion of amplicons with the single-strand-specific mung bean nuclease prior to T-RFLP analysis completely eliminated pseudo-T-RFs. This clearly indicates that single-stranded amplicons are the reason for the formation of pseudo-T-RFs, most probably because single-stranded restriction sites cannot be cleaved by restriction enzymes. The strong dependence of pseudo-T-RF formation on the number of cycles used in PCR indicates that (partly) single-stranded amplicons can be formed during amplification of 16S rRNA genes. In a model, we explain how transiently formed secondary structures of single-stranded amplicons may render single-stranded amplicons accessible to restriction enzymes. The occurrence of pseudo-T-RFs has consequences for the interpretation of T-RFLP profiles from environmental samples, since pseudo-T-RFs may lead to an overestimation of microbial diversity. Therefore, it is advisable to establish 16S rRNA gene sequence clone libraries in parallel with T-RFLP analysis from the same sample and to check clones for their in vitro digestion T-RF pattern to facilitate the detection of pseudo-T-RFs.

One of the most active fields in microbial ecology is the study of microbial communities in their natural habitats. Cultivation-independent molecular methods have become indispensable tools for this type of research, among which PCR is a core technique. Despite its known limitations (for a review, see reference 30), PCR amplification of 16S rRNA genes is an integral part of the so-called full-cycle rRNA analysis approach to community structure analysis (1), which involves cloning of amplified gene products, comparative sequence analysis of individual clones, and, subsequently, probe design and application of probes to environmental samples.

The caveats of the cloning approach (30), namely, the lack of analysis of a statistically significant number of clones required for complex communities, has encouraged the use of molecular techniques, which map the diversity of the community structure by PCR-based fingerprinting. In contrast to cloning analysis, fingerprinting techniques such as denaturing/thermal gradient gel electrophoresis (DGGE/TGGE) (for a review, see reference 19), single-stranded site conformational polymorphism (SSCP) (12, 25), and terminal restriction fragment length polymorphism (T-RFLP) (4, 13) (for reviews, see references 11 and 17) analyses allow the physical separation of the total pool of amplified community gene products.

Typically, T-RFLP analysis involves amplification of target genes from whole-community DNA extracts by using specific primer pairs, one of which is fluorescently labeled. Subsequently, amplicons are digested with restriction enzymes (usually tetranucleotide recognizing) and fragments are size separated via gel electrophoresis on automated sequencers, whereby only the labeled terminal fragments (T-RFs) are detected and quantified. Individual T-RFs can be assigned presumptively to operational taxonomic units, which ideally correspond to phylogenetically related microorganisms, based on in silico search for matching restriction sites in sequences from clone libraries established in parallel from the same sample. The 16S rRNA gene has been used extensively as marker gene for T-RFLP analysis (for reviews, see references 11 and 17).

In general, the T-RFLP technique has proven to be a reproducible and accurate tool for community fingerprinting (4, 13, 18, 22). Since T-RFLP analysis is based on PCR amplification, all biases related to this technique apply (30) and a number of important parameters related to PCR have been identified; it has been found that initial DNA template concentration, number of PCR cycles, annealing temperature, and the choice of *Taq* DNA polymerase from different manufacturers may affect the composition of T-RFLP profiles (4, 22).

T-RFLP-based gene ratios were found to be influenced by preferential gene amplification of specific templates (PCR drift

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[23, 28, 29]) when degenerated primers for the amplification of the *mcrA* (methyl coenzyme M reductase) gene were used (14, 16). On the other hand, Lueders and Friedrich (16) demonstrated that PCR–T-RFLP analysis can accurately reflect template ratios of archaeal 16S rRNA genes in a model community with defined amounts of 16S rRNA gene copies from five different methanogens.

In addition to PCR factors, the composition of T-RFLP profiles can be influenced by factors related to the restriction digestion, such as partially digested PCR products observed in T-RFLP profiles of pure cultures (3, 4) or environmental samples (22, 27). Additional restriction fragments (RFs) in T-RFLP profiles of pure cultures were attributed to either incomplete digestion of the amplicons or sequence heterogeneity of the template, i.e., multiple copies of 16S rRNA genes in single species with different terminal restriction sites (4). If the occurrence of additional peaks originates from incomplete digestion, this may be revealed under limiting restriction enzyme concentration (22). At any rate, incompletely digested PCR products from a complex microbial community may result in additional T-RFs and, consequently, an overestimation of diversity (22).

The present study was initiated to systematically examine the frequent occurrence of unexpected RFs in addition to the expected T-RFs after in vitro digestion of individual environmental 16S rRNA gene clones. These additional, nonterminal RFs in T-RFLP profiles were designated pseudo-T-RFs. Our results indicate that partially single-stranded amplicons are involved in the formation of pseudo-T-RFs.

MATERIALS AND METHODS

DNA extracts. DNA extracts and bacterial and archaeal 16S rRNA gene clones from gut compartments of cetoniid beetle (*Pachnoda ephippiata*) larvae are described elsewhere (M. Egert, T. Lemke, B. Wagner, A. Brune, and M. W. Friedrich, unpublished data). Accession numbers of clones used in this study are AJ538350 (clone PeM 75), AJ538351 (PeH59), and AJ538352 (PeMAr04). Pure-culture DNA extracts of *Methanococcus jannaschii* (DSM 2661^T), *Methanobacterium bryantii* (DSM 863^T), *Methanospirillum hungatei* (DSM 864^T), and *Methanosaeta concilii* (DSM 3671^T) were kindly provided by T. Lueders (MaxPlanck Institute, Marburg, Germany).

T-RFLP analysis. 16S rRNA genes were specifically amplified using the primer combination of 6-carboxyfluorescein (FAM)-labeled primers 27f (5′-AGA-GT T-TGA-TCC-TGG-CTC-AG-3′) (5) and 907r (5′-CCG-TCA-ATT-CCT-TTR-AGT-TT-3′) (20) for *Bacteria* and Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) (7) and FAM-labeled Ar912r (5′-CTC-CCC-CGC-CAA-TTC-CTT-TA-3′) (15) for *Archaea*. The standard reaction mixture contained, in a total volume of 50 μl, 1× PCR buffer II (Applied Biosystems, Weiterstadt, Germany), 1.5 mM $MgCl_2$, a 50 μM concentration of each of the four deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Freiburg, Germany), a 0.5 μM concentration of each primer (MWG Biotech, Ebersberg, Germany), and 1.25 U of Ampli *Taq* DNA polymerase (Applied Biosystems). In addition, 1 μl of a 1:30 dilution of *Pachnoda* gut DNA extract, 0.5 μl of a 1:10 dilution of clonal M13 product (including 16S rRNA gene sequence inserts), or 1 μl of pure-culture DNA extract was added as the template. All reaction mixtures were prepared at 4°C in 0.2-ml reaction tubes to avoid nonspecific priming. Amplification was started by placing the reaction tubes immediately into the preheated (94°C) block of a GeneAmp 9700 thermocycler (Applied Biosystems). The standard thermal profiles for the amplification of bacterial 16S rRNA genes were as follows: initial denaturation (94°C for 3 min) followed by 16 (clonal DNA templates) or 32 (environmental DNA templates) cycles of denaturation (94°C for 30 s), annealing (52°C for 30 s), and extension (72°C for 60 s). Thermal profiles for the amplification of archaeal 16S rRNA genes started with an initial denaturation (94°C for 3 min) followed by 16 (clonal and pure-culture DNA templates) or 35 to 38 (environmental templates) cycles of denaturation (94°C for 45 s), annealing (52°C for 45 s), and extension (72°C for 90 s). After terminal extension (72°C for 5 to 7 min), samples were stored at 4°C until further analysis. Aliquots (5 μl) of 16S rRNA amplicons were analyzed by gel electrophoresis on 1% agarose gels and visualized after being stained with ethidium bromide. PCR products were purified with the MinElute PCR purification kit (Qiagen, Hilden, Germany).

Prior to digestion, amplicon concentrations were determined photometrically. DNA (75 ng for amplicons from the gut DNA extract, 50 ng for clonal amplicons), 2.5 U of restriction enzymes (*Msp*I, *Taq*I, and *Alu*I [Promega, Mannheim, Germany]; *Msp*I, *Hpa*II, *Hha*I, *Hae*III, and *Bst*UI [New England Biolabs, Frankfurt am Main, Germany]; and *BsiS*I [Minotech Biotechnology, Heraklion, Crete, Greece]), 1 μl of 10× incubation buffer, and 1 μg of bovine serum albumin (if recommended) were combined in a total volume of 10 μl and digested for 3 h at 37°C (*Msp*I, *Alu*I, *Hpa*II, *Hha*I, and *Hae*III), 55°C (*Bsi*SI), 60°C (*Bst*UI), 65°C (*Taq*I), or 70°C (*Bsi*SI). Fluorescently labeled T-RFs were size separated on an ABI 373A automated sequencer (Applied Biosystems) using an internal size standard (GeneScan-1000 ROX; Applied Biosystems). T-RFLP electropherograms were analyzed with GeneScan 2.1 software (Applied Biosystems) (15).

Mung bean nuclease digest. The single-stranded DNA parts of 16S rRNA gene amplicons were digested using mung bean nuclease. Approximately 1,000 ng of PCR product was incubated for 1 h at 30°C with 5 U of mung bean nuclease (New England Biolabs) and 10 μl of 10× reaction buffer in a total volume of 100 μl. The digestion was stopped by phenol-chloroform-isoamyl alcohol (25:24:1) extraction, and DNA was recovered by ethanol precipitation. Digested amplicons were purified using the MinElute PCR purification kit.

RESULTS AND DISCUSSION

Occurrence of pseudo-T-RFs in T-RFLP analysis. Clones from two bacterial (109 clones) and two archaeal (68 clones) 16S rRNA gene clone libraries (established from DNA extracts of the midgut and hindgut of cetoniid beetle [*Pachnoda ephippiata*] larvae [Egert et al., unpublished]) were analyzed by PCR–T-RFLP using *Msp*I as the restriction endonuclease for the bacterial clones and *Alu*I as the restriction endonuclease for the archaeal clones. Theoretically, each clone was expected to display a single T-RF with a fragment length which is predictable from the respective sequence data. However, 50% of the bacterial and 78% of the archaeal clones reproducibly showed unexpected RFs in addition to the expected T-RF (examples are given in Fig. 1A and 2B). Moreover, additional RFs were observed for amplicons of two of four archaeal pure cultures (*Methanobacterium bryantii* and *Methanospirillum hungatei*) digested with *Alu*I as the restriction enzyme (Fig. 2C). Depending on the restriction enzyme used, single clones displayed either no, one, or (rarely) two or more additional RFs. Additional RFs occurred with a variety of restriction enzymes with different or identical recognition sites when multiple clones were analyzed, i.e., *Msp*I (from two different suppliers), *Hpa*II (an isoschizomer of *Msp*I), *Hha*I, *Bst*UI (e.g., clone PeM75 [Fig. 1]), *Alu*I (e.g., clones PeMAr04 [Fig. 2B] and PeH59 [Fig. 3B]), and *Hae*III (data not shown). In silico analysis of clonal and pure-culture sequence data revealed that all additional RFs corresponded to restriction sites downstream of the primary site (e.g., clone PeM75 [Fig. 1]). Thus, additional RFs most probably originated from a partial digestion of the amplicons in which the terminal restriction site was not cleaved.

For these additional, unexpected RFs, we introduce the name "pseudo-T-RFs" (Gr. adj. *pseudos,* meaning false; i.e., a false T-RF), because they are detectable as terminal, fluorescently labeled fragments in T-RFLP analysis; however, pseudo-T-RFs do not represent the actual ("real") terminal restriction

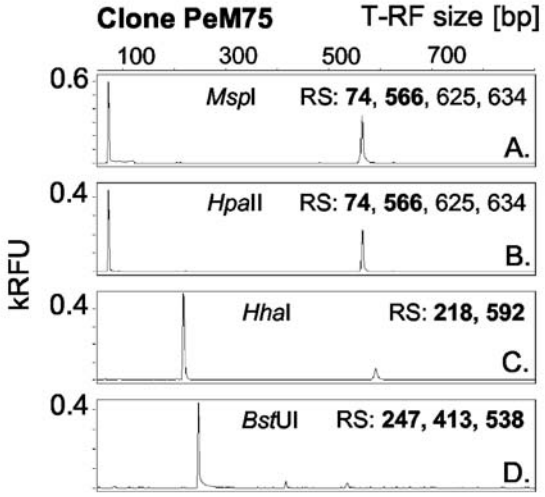

FIG. 1. Occurrence of pseudo-T-RFs in T-RFLP profiles of a single clone depending on the restriction enzyme used. 16S rRNA gene T-RFLP electropherograms were derived from clone PeM75 (affiliated with *Lactobacillales*). Numbers indicate restriction sites (RS) for the respective enzyme detected in the clonal sequence between bases 1 and ~900 (length of the PCR product), counted from the labeled 5′ end. Bold numbers indicate restriction sites with corresponding T-RFs in the electropherogram. RFU, relative fluorescence units.

fragment as predicted from sequence data and therefore have to be regarded as false T-RFs (hence, pseudo-T-RFs).

The frequent occurrence of pseudo-T-RFs in T-RFLP profiles of clones suggested their likely occurrence also in T-RFLP profiles of complex microbial communities. In fact, potential pseudo-T-RFs were identified in T-RFLP profiles of environmental samples, i.e., gut DNA extracts of *P. ephippiata* larvae and soil which was used for feeding the larvae (Egert et al., unpublished), by comparing predicted T-RFs of clones to those present in the mixed-community T-RFLP profile.

For example, the T-RFLP profile of archaeon-specific 16S rRNA gene amplicons from midgut DNA extracts with *Alu*I digestions was characterized by three T-RFs, two of which could be presumptively assigned to clonal sequences affiliated with the *Methanobacteriaceae* (T-RF of 64 bp; 6 clones) and *Crenarchaeota* (125 bp; 12 clones) (Fig. 2A). However, the prominent peak at 165 bp in the electropherogram was not reflected by any clone sequence; i.e., no clone sequence showed a primary, real terminal *Alu*I restriction site of 165 bp. In vitro digestion of clonal PCR amplicons revealed that all clones related to *Methanobacteriaceae* and *Crenarchaeota* displayed an additional 165-bp RF (shown in Fig. 2B for the crenarchaeotal clone PeMAr04). Therefore, it was assumed that the 165-bp T-RF in the midgut T-RFLP profile was a pseudo-T-RF.

Involvement of partly single-stranded amplicons in pseudo-T-RF formation. The occurrence of multiple RFs in T-RFLP profiles from single species has been reported for pure cultures (4, 6) and clonal PCR amplicons (6, 27), which were explained by 16S rRNA gene sequence heterogeneity, e.g., multiple rRNA operons in a single species (4), or partial digestion of the PCR products (4, 22, 27). Sequence heterogeneity of 16S rRNA genes can be excluded as a reason for the formation of pseudo-T-RFs, because we used amplicons of clonal origin. Nevertheless, a characteristic of all clones with pseudo-T-RFs was that the primary terminal restriction site was cleaved by the restriction enzyme for only a fraction of the amplicon pool; i.e., they were only partially digested (Fig. 1, 2B, and 3).

All efforts to overcome a bias related to partial digestion of amplicons were not successful. Use of twice as much enzyme (5 U) as in a typical digest (22) and extension of the digestion time (6 and 24 h) did not relieve the occurrence or the intensity of pseudo-T-RF peaks. It is noteworthy, though, that peaks with a size corresponding to full-length amplicons (~900 bp) were not present in T-RFLP profiles of clones with and without pseudo-T-RFs (e.g., Fig. 1, 3, and 4), which would have been indicative of incomplete digestion because of limiting enzyme concentration or suboptimal reaction conditions (22).

Since restriction endonucleases require double-stranded DNA at the restriction site (21), the presence of single-stranded amplicons in the range of the terminal restriction site was checked for by using mung bean nuclease, which degrades single-stranded DNA (10).

After mung bean nuclease digestion, pseudo-T-RFs were not detectable in environmental (*Pachnoda* gut), clonal, and pure-culture-derived T-RFLP profiles (Fig. 2). These data indicate clearly that the formation of pseudo-T-RFs results from the presence of at least partly single-stranded DNA amplicons. Single-stranded DNA is not a substrate for type II restriction endonucleases (21), and so the presence of single-stranded 5′-DNA ends of part of the amplicon pool—on otherwise double-stranded PCR products—provides an explanation of why the terminal restriction site was not cut. Similarly, mung bean nuclease treatment was used to remove single-stranded DNA artifacts prior to SSCP (10) and DGGE analysis (26).

By comparing T-RFLP patterns of individual clones with different restriction endonucleases, it became evident that the amplicon pool contains PCR products which are single stranded to different degrees. For example, *Bst*UI digestions of clone PeM75 amplicons yielded pseudo-T-RFs of 413 and 538 bp (Fig. 1D), which suggests that a small part of the amplicon pool is single stranded, at least up to the second *Bst*UI restriction site of PeM75 at bp 413.

The secondary structure of 16S rRNA gene sequences influences restriction digests. Some clones displayed pseudo-T-RFs with one enzyme but not with the other when amplicons from the same PCR batch were analyzed by T-RFLP. For example, clone PeH59 had a primary *Msp*I restriction site at 81 bp and eight subsequent restriction sites as revealed by sequence data analysis (Fig. 3A), but pseudo-T-RFs were not formed. Digests with *Alu*I (Fig. 3B), *Hha*I (Fig. 3C), and *Bst*UI (data not shown) revealed pseudo-T-RFs up to 638 bp (*Alu*I [Fig. 3B]), which suggests that some amplicons were single stranded at least up to bp 241. According to a model which involves the formation of transiently formed secondary structures composed of recognition sequences with twofold rotational symmetry ("canonical structures"), many type II restriction endonucleases cleave single-stranded DNA (21). Inspection of possible secondary structures as calculated with the program *mfold* (24) (M. Zuker; http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) showed that the primary *Msp*I restriction site

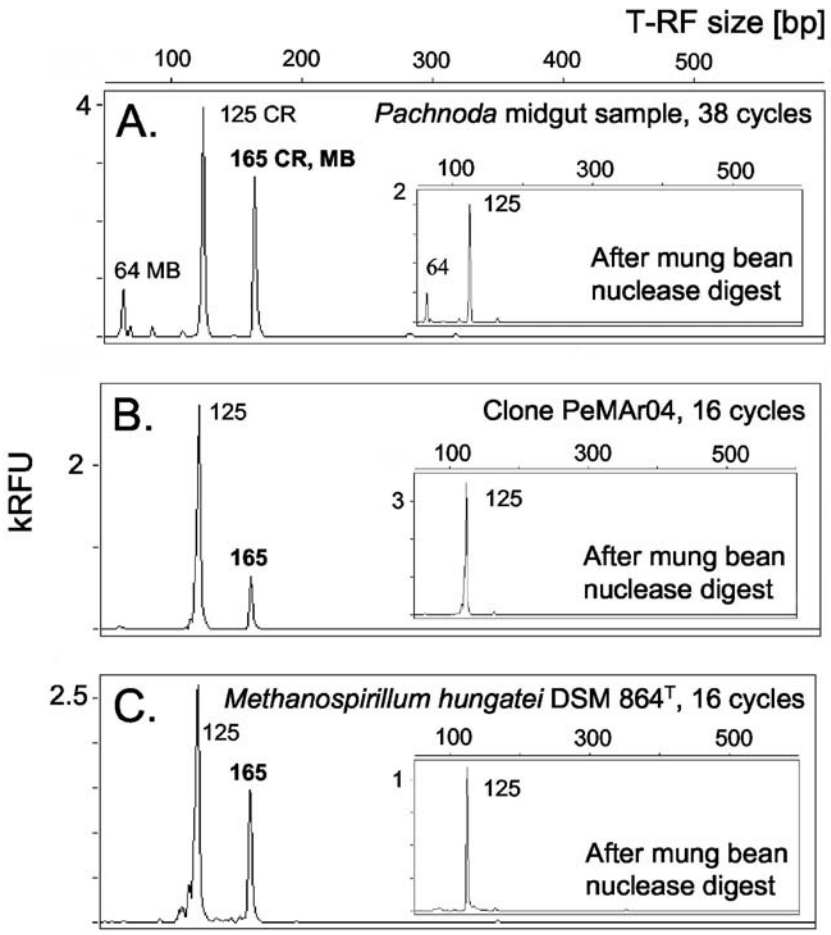

FIG. 2. Effect of mung bean nuclease digestion on the occurrence of pseudo-T-RFs in T-RFLP profiles (*Alu*I digests) of environmental, clonal, and pure-culture samples. Insets show the T-RFLP profile after mung bean nuclease digestion. The number of PCR cycles used to produce the amplicons is indicated. Fragment lengths of pseudo-T-RFs are shown in bold. Clone PeMAr04 is affiliated with the kingdom *Crenarchaeota.* MB, *Methanobactericeae;* CR, *Crenarchaeota;* RFU, relative fluorescence units.

of clone PeH59 was able to form a canonical structure (i.e., a local secondary structure) by folding back with an upstream single-stranded sequence (Fig. 3D). Although the secondary structures did not form a perfect palindrome, it is likely that the primary restriction site of single-stranded amplicons was indeed cleaved by *Msp*I, since pseudo-T-RFs downstream from the primary restriction site were not detected. In contrast, the primary *Alu*I restriction site of clone PeH59 most probably did not form a sterically sufficient secondary structure from single-stranded DNA, and thus *Alu*I did not cleave single-stranded amplicons at the primary recognition site, which corroborates the presence of a pseudo-T-RF at 638 bp. It should be noted, however, that the predicted secondary structures represent the most thermodynamically stable structures according to the underlying model (24) as implemented in *mfold;* thus, these structures may actually not exist in the reaction mixture of the T-RFLP digest. However, restriction digests conducted at different temperatures provide experimental evidence that canonical structures in single strands might be the reason why some clonal amplicons do not show pseudo-T-RF formation with certain nucleases. Clone PeH59 did not show pseudo-T-RFs when digested with *Msp*I at 37°C (Fig. 3A) or *Bsi*SI at 55°C (Fig. 4A); *Bsi*SI is an isoschizomer of *Msp*I which is not inactivated by heat. However, at 70°C (Fig. 4B), pseudo-T-RFs occurred when *Bsi*SI was used and were even more pronounced when the amplicons were denatured for 3 min at 94°C prior to digestion (Fig. 4C). At increased digestion temperature, canonical structures in single-stranded amplicons are likely to become unstable, rendering the restriction sites inaccessible to the nuclease, which in turn leads to the formation of pseudo-T-RFs. Interestingly, pseudo-T-RFs were not detectable using *Taq*I as the restriction endonuclease at a digestion temperature of 65°C, even with clones that possessed multiple *Taq*I restriction sites and displayed pseudo-T-RFs with other

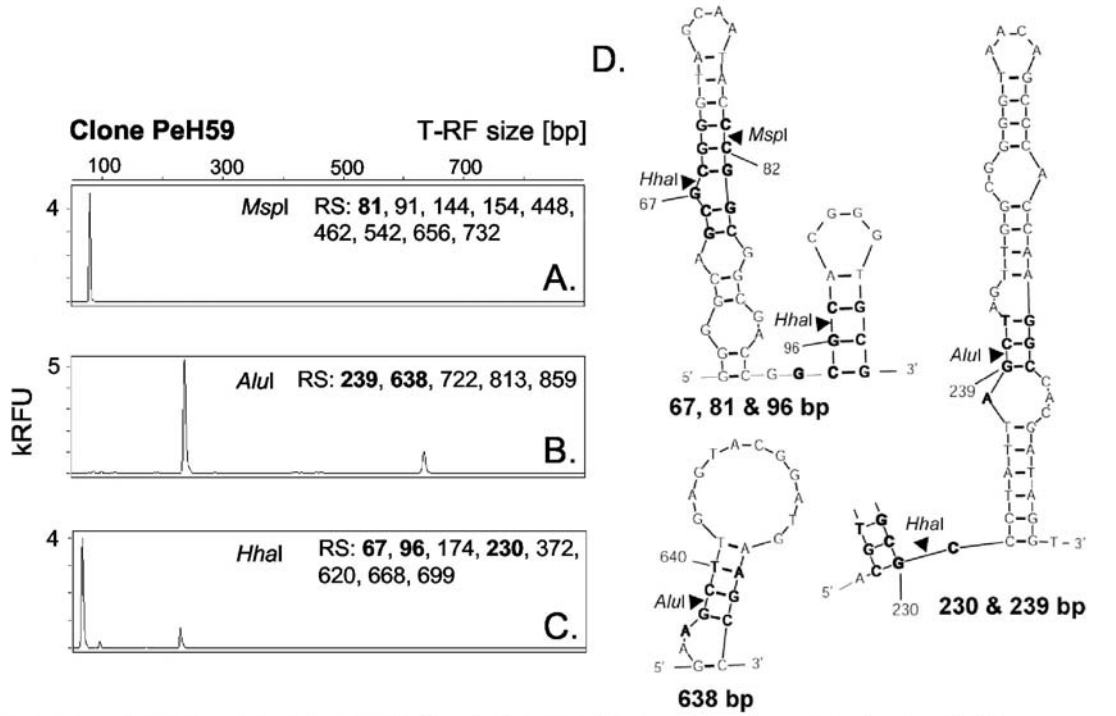

FIG. 3. (A to C) T-RFLP analysis of clone PeH59 (affiliated with the CFB phylum) amplicons after restriction digestion with different enzymes, resulting in the expected T-RFs only (*Msp*I [A]) or in the formation of pseudo-T-RFs (*Alu*I [B] and *Hha*I [C]). (D) 16S rRNA gene secondary structure of clone PeH59 as predicted by the *mfold* software including the sequence stretches around detected pseudo-T-RFs. RS, restriction sites. Bold numbers indicate restriction sites with corresponding T-RFs in the electropherogram. RFU, relative fluorescence units.

nucleases (tested only for archaeal clones [data not shown]). Possibly, *Taq*I cleaves single-stranded amplicons not involved in canonical structures at a higher rate than the other restriction enzymes analyzed, making *Taq*I suitable as an endonuclease that avoids formation of pseudo-T-RFs in T-RFLP analysis.

In general, the extent of pseudo-T-RF formation (for all restriction endonucleases tested) decreased with increasing distance of the terminal restriction site from the 5′ labeled end of the amplicon (Fig. 5), which supports the hypothesis that amplicons are partly single stranded.

Influence of PCR on the formation of pseudo-T-RFs. We found that the height and area of a pseudo-T-RF in relation to those of the primary, expected RF increased with the number of PCR cycles used to produce the amplicon (Fig. 6). This shows clearly that the occurrence of pseudo-T-RFs is a PCR artifact and that a PCR bias is apparently involved in the formation of partly single-stranded amplicons. Therefore, we tried to optimize the PCR protocols, but none of the following modifications, tested with selected bacterial clones, significantly affected pseudo-T-RF formation: (i) prolonged extension (1 min 30 s or 2 min) or final extension time (10 or 15 min), (ii) increased concentration of Ampli *Taq* DNA polymerase (3 U) or addition of fresh polymerase (3 U) before the final extension step to exclude polymerase limitation, (iii) addition of *Pfu* DNA polymerase (Promega) with proofreading activity, (iv) increased concentration of primers (1 μM each), (v) decreased initial template concentration (~100 to 10^{-3} ng μl^{-1}), or (vi) higher (i.e., more stringent) annealing temperatures (55, 57, 59, 61, or 64°C) to determine whether the formation of single-stranded DNA could result from incorrectly annealed primers. Unexpectedly, at annealing temperatures of 61 and 64°C, the number of pseudo-T-RFs even increased. Lower annealing temperatures (50, 48, and 46°C) did not affect the formation of pseudo-T-RFs.

The formation of single-stranded amplicons can be favored by a differential, asymmetric utilization of primers in the PCR amplification due to differences in priming efficiencies, which can result from differences in the G+C content of the primers used (10). Therefore, primer concentrations were varied at ratios of 1:8 to 8:1 (27f versus 907r; G+C content, 50 and 37.5%, respectively) to overcome a possible bias related to asymmetric primer utilization in the PCR, but the formation of pseudo-T-RFs was unaffected.

The formation of partly single-stranded 16S rRNA gene amplicons during PCR may result from template secondary structures (10), which causes the polymerase to pause or fall off the template (23). However, use of the PCR enhancer betaine at various concentrations, which had been shown to be effective in improving the amplification yield and the specificity of

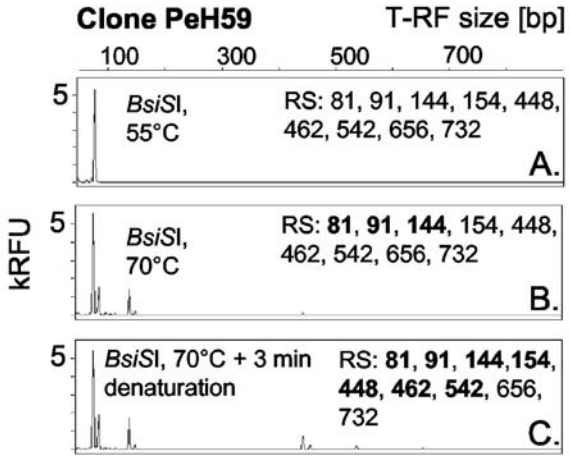

FIG. 4. Effect of restriction digest temperature on the formation of pseudo-T-RFs of clone PeH59. Restriction digests were performed using *Bsi*SI at 55°C (A) and 70°C (B) and by using a 3-min denaturation of the PCR amplicon prior to the addition of enzyme and incubation at 70°C (C). Bold numbers indicate restriction sites with corresponding T-RFs in the electropherogram. RFU, relative fluorescence units.

templates with high G+C content or secondary structures (9), did not prevent the formation of pseudo-T-RFs.

Model for the formation of pseudo-T-RFs. Based on the above results, we propose the following model for the formation of pseudo-T-RFs during T-RFLP analysis of 16S rRNA genes. During PCR of 16S rRNA genes from clonal, pure-culture, and environmental DNA extracts, some of the amplicons formed are at least partly single stranded (as proven by mung bean nuclease digests [Fig. 2]). Since single-stranded terminal restriction sites cannot be cleaved by restriction endonucleases, "pseudo"-terminal restriction sites downstream from the expected primary restriction site can be detected by T-RFLP analysis. The ability of the 16S RNA molecule to backfold with itself (8) may result in an incomplete synthesis of a fraction of 16S rRNA gene amplicons during PCR (Fig. 7A). The involvement of PCR in the generation of (partly) single-stranded amplicons is corroborated by the strong dependence on the number of PCR cycles (Fig. 6). Similarly, the number of PCR cycles has been implicated as a controlling factor in a kinetic model which describes the reannealing of single-stranded templates as a source of the PCR bias (28). Accordingly, the formation of single-stranded amplicons may be viewed as an extension of the original kinetic model of template reannealing: when the amplicon concentration increases at greater PCR cycle numbers, the rate of interaction between single-stranded template molecules increases, which may result not only in interstrand reannealing as described by Suzuki and Giovannoni (28) but also in intrastrand annealing, hence the formation of local secondary structures. In turn, these temporary secondary structures of template molecules may cause the DNA polymerase molecules to fall off with higher frequency (23), thereby leaving the template strands (partially) unamplified. Furthermore, we hypothesize that single-stranded 16S rRNA gene amplicons can form local palindromic secondary structures, which in turn allow restriction enzymes to cut "single-stranded" DNA (21). This hypothesis helps explain why T-RFLP analyses with certain enzymes yield pseudo-T-RFs whereas others from the same PCR amplification do not (Fig. 7B): a secondary restriction site will be detected in T-RFLP analysis only if the primary restriction is not part of a canonical structure. We could show that higher temperatures (70°C, [Fig. 4B and C]) during restriction digestion resulted in the formation of pseudo-T-RFs, most probably because local secondary structures were unstable under these conditions and consequently were no longer substrates for the restriction enzyme. Thus, the sequence context around the primary restriction site

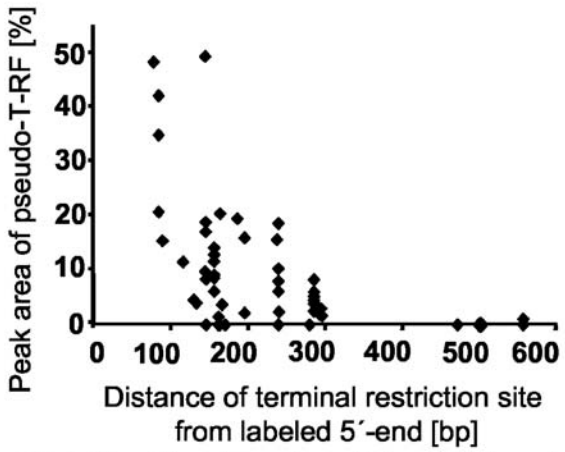

FIG. 5. Effect of the position of the terminal restriction site on the extent of pseudo-T-RF formation, based on in vitro T-RF formation of 56 bacterial clones with *Msp*I as the restriction endonuclease. The peak area of the pseudo-T-RF is compared to the peak area of the primary T-RF and given as a percentage. Clones were obtained from a 16S rRNA gene clone library derived from the midgut of cetoniid beetle larvae (Egert et al., unpublished).

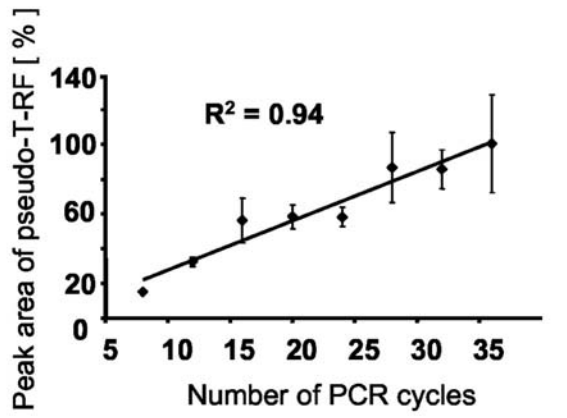

FIG. 6. Effect of PCR cycle number on the extent of pseudo-T-RF formation observed with amplicons of clone PeM75 after *Msp*I digestion. The peak area of the pseudo-T-RF is compared to the peak area of the primary T-RF and given as a percentage. Error bars (which represent standard deviation) are based on three replicates.

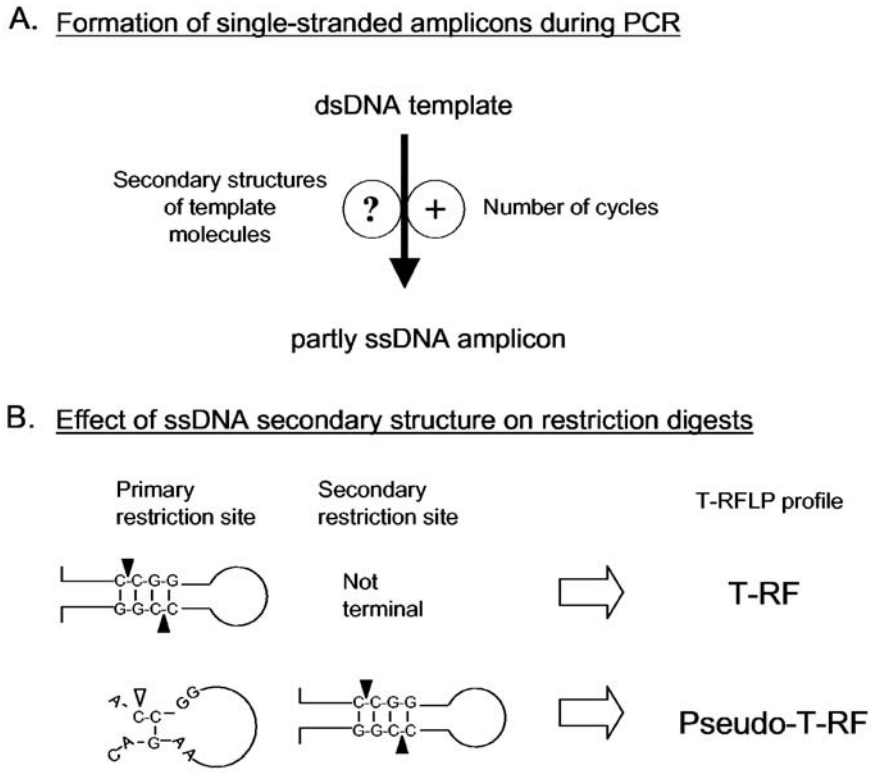

FIG. 7. Schematic model of pseudo-T-RF formation. (A) PCR-related parameters influencing the formation of partly single-stranded amplicons. (B) Involvement of the secondary structure of partly single-stranded amplicons in the formation of pseudo-T-RFs. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; solid triangles, restriction site cut (*Msp*I); open triangle, restriction site not cut.

most probably will determine whether even a single-stranded amplicon can be digested at its primary, real terminal restriction site.

Regardless of the underlying mechanism for secondary-structure formation, pseudo-T-RFs occurred in 16S rRNA gene clones from diverse phylogenetic lineages (i.e., *Lactobacillales, Bacillales, Clostridia,* high-G+C gram-positive *Bacteria, Crenarchaeota,* and *Euryarchaeota* [Egert et al., unpublished]), which shows that most 16S rRNA gene amplicons may be affected. Interestingly, pseudo-T-RFs were not detected in T-RFLP analysis of cloned *nirK* genes (copper-containing nitrite reductase [2; G. Braker, personal communication]) or *mcrA* genes (methyl-coenzyme M reductase [T. Lueders, personal communication]); apparently, PCR products of these genes do not form secondary structures which are as thermodynamically stable as those formed in 16S rRNA molecules.

Conclusions and recommendations. The occurrence of pseudo-T-RFs in T-RFLP profiles has consequences for the interpretation of the underlying microbial diversity. First of all, if pseudo-T-RFs are not identified, diversity may be overestimated because of the larger number of peaks in T-RFLP profiles. For example, in the *Alu*I-based archaeon-specific T-RFLP analysis of the *Pachnoda* midgut, the prominent T-RF of 165 bp (Fig. 2A) did not represent additional diversity which was overlooked by clone library analysis but, rather, could be clearly identified as a pseudo-T-RF originating from clone sequences related to *Methanobacteriaceae* and *Crenarchaeota.* Second, curing of pseudo-T-RFs by simple elimination through mung bean nuclease digestion (Fig. 2A) will result in an underestimation of the relative gene frequency of amplicons which are affected by the formation of pseudo-T-RFs; in the case of the prominent archaeal pseudo-T-RF of 165 bp, 26% of the total 16S RNA gene frequency was represented by the pseudo-T-RF. When the in vitro digestion pattern of clones was tested, the number of assignable T-RFs in a *Bacteria*-specific T-RFLP profile from the hindgut of *Pachnoda* larvae (Egert et al., unpublished) increased from 18 to 27. To this end, it should be kept in mind that the restriction enzyme for T-RFLP analysis which produces the largest number of peaks from a given amplicon pool may not be the most suitable one, because the increase in the number of T-RF peaks may be a reflection of an increased number of pseudo-T-RFs only.

Since the extent of pseudo-T-RF formation is likely to be dependent on the species (gene) composition of the system under investigation and the chosen restriction endonuclease(s), it is advisable to perform T-RFLP analysis and cloning in parallel. Although this results in increased effort, the T-RF patterns of clones should be determined by in vitro T-RFLP

analysis under the applied PCR and T-RFLP conditions, in particular when T-RFs are supposed to be quantitatively assigned to species or phylogenetic groups. Assigning T-RFs solely on the basis of in silico or database search is insufficient because of the potential occurrence of pseudo-T-RFs in T-RFLP profiles. In agreement with several other studies (22, 23, 28, 29), the number of PCR cycles should be limited to a minimum because pseudo-T-RF formation increases linearly with the cycle number. Beyond T-RFLP fingerprinting, the formation of (partly) single-stranded 16S rRNA gene amplicons during PCR may also affect other core techniques in microbial ecology, e.g., 16S rRNA gene cloning. In 16S rRNA gene clone libraries, sequences with a strong tendency to produce single-stranded amplicons are likely to be underrepresented because the single-stranded fraction of the amplicons cannot be ligated into the cloning vector.

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3.9 Einsatz von Klenow-Fragment zur Vermeidung von pseudo-T-RFs

Als Klenow-Fragment bezeichnet man ein durch proteolytische Spaltung entstehendes Fragment aus der DNA-Polymerase I von *E. coli*. Im Gegensatz zum Gesamtenzym besitzt das Klenow-Fragment keine 5´-3´-Exonuklease mehr, sondern nur noch die 5´-3´-Polymerase und eine 3´-5´-Exonuklease Aktivität. In der Biotechnologie findet es u.a. Verwendung bei der Zweitstrangsynthese einzelsträngiger DNA oder zur Erzeugung von DNA-Molekülen mit stumpfen Enden (blunt ends) aus solchen mit überhängenden Enden (sticky ends).

Unter der Annahme, dass (an ihrem 5´-Ende) partiell einzelsträngige DNA-Amplikons die Ursache für die Entstehung von pseudo-T-RFs sind (s. Kapitel 3.8), wurde Klenow-Fragment eingesetzt, um diese quasi als lange 5´-Überhänge anzusehenden Amplikonenden mit Basen aufzufüllen und so die terminalen Schnittstellen aller Amplikons zugänglich für Restriktionsenzyme zu machen. Dabei wurde, um grundsätzlich jede Form einer Exonuklease-Aktivität ausschließen zu können, eine käufliche Variante des Klenow-Fragments eingesetzt, die durch eine Mutation auch keine 3´-5´-Exonuklease-Aktivität mehr besitzt.

Der Effekt einer der T-RFLP-Analyse vorgeschalteten Klenow-Behandlung des Amplikonpools wurde mit einem artifiziellen Gemisch zweier klonaler M13-Produkte (ca. 5 ng μl^{-1}) als PCR-Template getestet (Standard-PCR mit 32 Zyklen). Während einer der beiden eingesetzten Klone (PeH 55, *Lactobacillales*) pseudo-T-RF-Bildung zeigte (565 bp pseudo-T-RF zusätzlich zum 72 bp-T-RF; Abb. 3.4), zeigte der andere Klon (PeH59; CFB-Phylum) erwartungsgemäß nur ein T-RF (81 bp), wenn *Msp*I als Restriktionsenzym verwendet wurde (s. Kapitel 3.8).

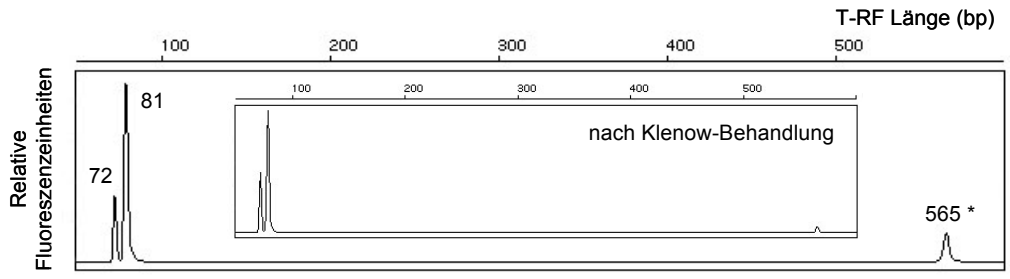

Abbildung 3.4: Exemplarische T-RFLP-Profile von 16S rRNA-Genen des untersuchten Klongemisches vor und nach einer Behandlung mit Klenow-Fragment. Restriktionsenzym: *Msp*I. * markiert das pseudo-T-RF.

Es wurde erwartet, dass die Klenow-Behandlung - im Gegensatz zur Behandlung mit Mung bean-Nuklease, die einzelsträngige Amplikons lediglich abbaut - nicht nur zu einem Verschwinden (bzw. einer deutlichen Verringerung) der pseudo-T-RF Peaks (hier: 565 bp Peak) führt, sondern gleichzeitig eine Vergrößerung des Peaks bewirkt, der für die pseudo-T-RFs verantwortlich ist (hier: 72 bp-Peak). Im Idealfall sollte der 72 bp-Peak um den Betrag wachsen, um den der 565 bp-Peak kleiner wird. Da aus T-RFLP-Analysen nur relative Veränderungen ableitbar sind, wurde als Prüfgröße das Verhältnis der Peakhöhen des 81 bp-T-RFs zum 72 bp-T-RF untersucht, im Folgenden kurz als 81/72-Verhältnis bezeichnet. Es sollte unter Klenow-Behandlung abnehmen, wenn der 72er-Peak relativ zum 81er-Peak wächst. Um abzusichern, dass beobachtete Effekte tatsächlich auf einer Polymeraseaktivität des Klenow-Fragments beruhen, wurden Amplikons auch ohne Klenow-Fragment (aber mit dNTPs und Klenow-Puffer) sowie mit ddNTPs statt dNTPs inkubiert. Der Einsatz von ddNTPs sollte über Kettenabbrüche eine Polymeraseaktivität des Klenow-Fragments verhindern. Zur statistischen Absicherung wurden alle Versuche mit 3 - 4 Wiederholungen durchgeführt und mit U-Tests (Mann-Whitney) paarweise auf ihre Signifikanz ($P < 0,05$) hin untersucht. Die Ergebnisse zeigen Abbildung 3.4 und Tabelle 3.2.

Tabelle 3.2: Veränderung des 81/72-Verhältnisses (s. Text) in T-RFLP-Profilen von 16S rRNA-Genen eines artifiziellen Klongemisches unter verschiedenen, der T-RFLP-Analyse vorgeschalteten Behandlungen des Amplikonpools (Mittelwerte ± Standardfehler; n = 3 - 4).

Behandlung	**81/72-Verhältnis**
keine	2,6 ± 0,1
berechnete pseudo-T-RF-Umwandlung (s. Text)	1,9 ± 0,1
Klenow-Fragment Exonuklease minus	2,1 ± 0,1
Klenow-Puffer + dNTPs	2,9 ± 0,1
Klenow-Fragment Exonuklease minus + ddNTPs	2,4 ± 0,1
Mung bean-Nuklease	3,4 ± 0,3

Schlägt man rein rechnerisch die Höhe des 565 bp pseudo-T-RFs aus den Profilen unbehandelter Amplikons der Höhe des 72 bp-T-RFs zu, so sollte sich das 81/72-Verhältnis im Mittel von 2,6 : 1 auf 1,9 : 1 verringern (Tabelle 3.2). Nach einer Behandlung mit Klenow-Fragment

war der 565 bp-pseudo-T-RF-Peak zwar nie völlig verschwunden, aber im Mittel um ca. 75% kleiner als in Profilen unbehandelter Amplikons (Abb. 3.4). Gleichzeitig sank das 81/72-Verhältnis signifikant auf 2,1 : 1 ab. Zwischen dem experimentell ermittelten 81/72-Verhältnis nach Klenow-Behandlung und dem theoretisch erwarteten Verhältnis nach völliger Umwandlung der pseudo-T-RFs bestand dagegen kein signifikanter Unterschied. Die Inkubation von Amplikons ohne Klenow-Fragment oder mit ddNTPs war zum einen ohne erkennbaren Einfluss auf die Höhe des 565 bp-pseudo-T-RFs (konstant bei knapp 40% der Höhe des 72 bp-T-RFs) und führte auch nicht zu einer signifikanten Verringerung des 81/72-Verhältnises. Eine Behandlung mit Mung bean-Nuklease verkleinerte den 565 bp-Peak im Mittel um ca. 85 % und war (unerwartet) auch mit einer signifikanten Veränderung des 81/72-Verhältnises verbundenen, allerdings mit einem Anstieg auf 3,4 : 1 (d.h., dass der 72 bp-Peak relativ kleiner wurde).

Nach den erfolgreichen Versuchen mit einem Klongemisch wurden zusätzliche Versuche mit einer Umweltprobe durchgeführt. Hierzu wurden T-RFLP-Profile (Standard-PCR mit 38 Zyklen) der archaeellen Gemeinschaft im Mitteldarm von *P. ephippita* Larven nach verschiedenen Behandlungen miteinander verglichen. Diese Profile zeigen 3 dominante T-RFs, die den zwei im Mitteldarm von *Pachnoda* detektierten *Archaea*-Gruppen zuzuordnen sind (Abb. 3.5): 64 bp-T-RF (*Methanobacteriaceae*), 125 bp-T-RF (*Crenarchaeota*) und 165 bp-pseudo-T-RF (*Methanobacteriaceae* und *Crenarchaeota*).

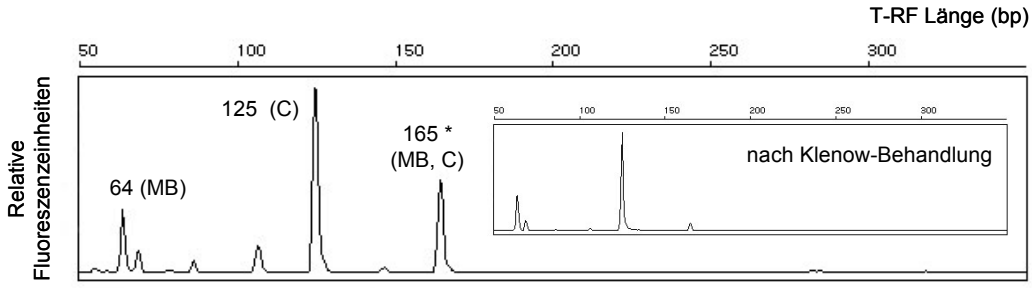

Abbildung 3.5: Exemplarische T-RFLP-Profile von 16S rRNA-Genen der *Archaea*-Gemeinschaft im *Pachnoda* Mitteldarm vor und nach einer Behandlung mit Klenow-Fragment. Restriktionsenzym: *Alu*I. * markiert das pseudo-T-RF.

Um abzuschätzen, zu welchen Teilen das 165 bp-T-RF den beiden Gruppen jeweils zuzurechnen ist, wurde je ein Klon aus beiden Gruppen (PeMAr19 und PeMAr04) auf sein in-vitro T-RF-Bildungsverhalten hin getestet (Standard-PCR mit 38 Zyklen und klonalem M13-Produkt

(ca. 5 ng μl^{-1}) als Matrizen-DNA). Während der untersuchte *Methanobacteriaceae*-Klon ein pseudo-T-RF mit 26 ± 0,9% (n = 5) der Höhe des terminalen Fragmentes (64 bp) zeigte, erreichte dieses beim *Crenachaeota*-Klon nur eine Höhe von 14 ± 0,9 % der Höhe des 125 bp-T-RFs. Erwartungsgemäß zeigte also der Klon, dessen terminale Schnittstelle (*Alu*I) näher am markierten 5´-Ende des Amplikons lag (PeMAr19, *Methanobacteriaceae*) auch die stärkere pseudo-T-RF-Bildung (Kapitel 3.8).

Tabelle 3.3 zeigt nun die Veränderung des Peakhöhen-Verhältnisses des 125 bp-T-RFs (*Crenarchaeota*) zum 64 bp-T-RF (*Methanobacteriaceae*) (kurz: 125/64-Verhältnis) in T-RFLP-Profilen nach verschiedenen Behandlungen (jeweils 5 - 7 Parallelen pro Behandlung) des Amplikonpools. Das zu erwartende 125/64-Verhältnis nach Überführung des 165 bp-pseudo-T-RFs wurde unter Berücksichtigung des in in-vitro T-RF Bildungsverhalten der beiden getesteten Klone abgeschätzt: Hierzu wurde, ausgehend von unbehandelten Profilen, die Höhe des 64 bp-T-RFs durch 0,74 und die des 125 bp-T-RFs durch 0,86 geteilt und anschließend das „neue“ Verhältnis berechnet. Damit wurde berücksichtigt, dass in unbehandelten Profilen der 64 bp-Peak offensichtlich nur 74% und der 125 bp-Peak nur 86% seiner eigentlichen Höhe zeigen, weil der Rest im 165 bp-pseudo-T-RF-Peak aufgeht (s.o.). Aufgrund dieser Abschätzung wurde erwartete, dass durch eine Klenow-Behandlung das 125/64-Verhältnis sinken sollte, da der stärker pseudo-T-RF beeinflusste 64 bp-Peak gegenüber dem 125 bp-Peak stärker an Höhe zunehmen sollte.

Tabelle 3.3: Veränderung des 125/64-Verhältnises (s. Text) in 16S rRNA-Gen T-RFLP-Profilen der *Archaea*-Gemeinschaft im Mitteldarm von *P. ephippiata* Larven unter verschiedenen, der T-RFLP-Analyse vorgeschalteten Behandlungen des Amplikonpools (Mittelwerte ± Standardfehler; n = 5 - 7).

Behandlung	**125/64-Verhältnis**
unbehandelt	3,4 ± 0,4
berechnete pseudo-T-RF-Umwandlung (s. Text)	2,9 ± 0,3
Klenow-Fragment Exonuklease minus	2,6 ± 0,2
Mung bean-Nuklease	3,6 ± 0,5

Eine Behandlung des Amplikonpools mit Klenow-Fragment vor der T-RFLP-Analyse führte nahezu zu einem Verschwinden des 165 bp-T-RFs (Abb. 3.5) und zeigte im Mittel die erwartete Verringerung des 125/64-Verhältnisses. Die statistische Analyse zeigte, dass das 125/64-Verhältnis nach Klenow-Behandlung mit eingeschränkter Signifikanz ($P < 0.10$) niedriger war als in den unbehandelten Proben, wohingegen zwischen dem erwarteten und experimentell bestimmten „neuen“ Verhältnis kein signifikanter Unterschied nachzuweisen war. Eine Behandlung mit Mung bean-Nuklease führte auch zu einem nahezu vollständigen Verschwinden des 165 bp-pseudo-T-RFs (Kapitel 3.8), allerdings ohne einen signifikanten Effekt auf das 125/64-Verhältnis zu haben (Tab. 3.3).

Sowohl die Ergebnisse mit einem bakteriellen Klongemisch als auch mit archaeellen T-RFLP-Profilen aus einer Umweltprobe deuten darauf hin, dass durch die Behandlung mit Klenow-Fragment tatsächlich ein Großteil der partiell einzelsträngigen und dadurch pseudo-T-RFs verursachenden Amplikons mit Nukleotiden so aufgefüllt werden konnte, dass die terminalen Schnittstellen in ihnen für ein Restriktionsenzym wieder zugänglich wurden. Eine der T-RFLP-Analyse vorgeschaltete Behandlung des Amplikonpools mit Klenow-Fragment erscheint daher zum einen als probates Mittel zur Identifikation von pseudo-T-RFs, da diese dadurch deutlich verkleinert werden. Im Gegensatz zu einer Behandlung mit Mung bean-Nuklease, die dies auch leistet, können die so generierten T-RFLP-Profile aber anschließend auch zu einer quantitativen Auswertung benutzt werden, da die pseudo-T-RFs nicht nur eliminiert, sondern in „echte“ T-RFs überführt wurden, was ein Mung bean-Nuklease-Verdau nicht zu leisten vermag.

4 Zusammenfassende Diskussion

Boden-Invertebraten und ihre Darmflora sind entscheidend an der Transformation organischer Substanz in Böden beteiligt, deren Qualität und Quantität wiederum für viele Schlüsselfunktionen des Bodens verantwortlich ist. Im Rahmen dieser Arbeit wurden Struktur und räumliche Verteilung mikrobieller Gemeinschaften (v.a. *Bacteria* und *Archaea*) im Verdauungstrakt humivorer und phytophager Scarabaeidenlarven (*Pachnoda ephippiata* bzw. *Melolontha melolontha*) sowie von Regenwürmern (*Lumbricus terrestris*) mit Methoden der molekularen mikrobiellen Ökologie (Klonierung und phylogenetische Analyse von 16S rRNA-Genen, T-RFLP-Analyse, FISH) untersucht. Ziel war, über Vergleiche der mikrobiellen Gemeinschaften im gefressenen Futter, in verschiedenen Darmabschnitten sowie in verschiedenen Fraktionen eines Darmabschnittes (Darmwand und -lumen) zu einem tieferen Verständnis der Funktionen zu gelangen, die Mikroorganismen bei der Verdauung haben. Darüber hinaus wurde im Rahmen dieser Arbeit ein PCR-Artefakt entdeckt – die Bildung sog. pseudo-T-RFs – der große Auswirkungen für die T-RFLP-Analyse mikrobieller Gemeinschaften hat, eine in der molekularen mikrobiellen Ökologie mittlerweile sehr häufig eingesetzte „community-fingerprint"-Methode.

4.1 Struktur und Topologie mikrobieller Gemeinschaften im Darm von Scarabaeidenlarven

Scarabaeidenlarven – neue Modellsysteme für intestinale Transformationsprozesse

Bislang galten Termiten, und hier insbesondere die Holz fressenden Arten, als die Boden-Invertebraten, an denen die Interaktionen mit (intestinalen) Mikroorganismen bei der Transformation organischer Substanz am Besten untersucht und verstanden sind (Breznak, 2000; Brune, 2003; Ohkuma, 2003). Dies erklärt sich u.a. mit den hohen Biomasse-Abundanzen, die Termiten in ihren Verbreitungsgebieten erreichen können, und der daraus abgeleiteten großen Bedeutung dieser Tiere für den C- und N-Kreislauf in tropischen und subtropischen Ökosystemen (s. Kapitel 1.2; auch (Nardi et al., 2002)). Für ein tieferes Verständnis des Zusammenwirkens von Boden-Invertebraten mit Mikroorganismen bei der Stabilisierung und Mineralisierung organischer Substanz im Boden ist es allerdings notwendig, den Kreis untersuchter

Boden-Invertebraten auf andere Modellsysteme zu erweitern, zumal Termiten in Boden-Ökosystemen gemäßigter Breiten ohne Bedeutung sind. Aus dem Kreis der Boden-Makroinvertebraten kommen hier z.B. Ameisen, Asseln, Diplopoden, Dipterenlarven, Käferlarven und Regenwürmer in Betracht.
Die Larven („Engerlinge“) von Scarabaeiden (Blatthornkäfern), einer weltweit verbreiteten Käferfamilie mit geschätzten 25.000 Arten (Klausnitzer, 2002), bieten sich als Modellsysteme zum Studium intestinaler Transformationsprozesse an, da sie im Vergleich zu anderen Boden-Invertebraten zumindest ansatzweise mikrobiologisch untersucht sind (s. Kapitel 1.3). Unter ihnen findet man Vertreter verschiedenster Ernährungstypen (saprophag, humivor, coprophag, phytohag), darunter einige wirtschaftlich sehr bedeutende Pflanzenschädlinge, wie z.B. die in dieser Arbeit untersuchten phytophagen Larven von *Melolontha melolontha* (Keller, 1986). Der Intestinaltrakt aller Scarabaeidenlarven gliedert sich in zwei Hauptabschnitte: einen alkalischen, mit hydrolytischen Enzymaktivitäten ausgestatteten Mitteldarm und einen eher neutralen, als Gärkammer angesehenen Enddarm, die beide dicht mit Populationen von Mikroorganismen besetzt sind (Cazemier et al., 1997a). Über die genaue Zusammensetzung dieser mikrobiellen Populationen oder ihre räumliche Verteilung innerhalb des Verdauungstraktes war bislang kaum etwas bekannt. Insbesondere die Topologie mikrobieller Gemeinschaften wird aber als unerlässliche Vorrausetzung für ein tieferes Verständnis der Funktion mikrobieller Gemeinschaften im Darm von Insekten angesehen, die durch ausgeprägte Gradienten physiko-chemischer Parameter charakterisiert sind (Brune, 1998; Brune und Friedrich, 2000).

Die im Rahmen dieser Arbeit durchgeführten Untersuchungen bakterieller und archaeeller Diversität im Darm von *Pachnoda ephippiata* (Kongo-Rosenkäfer) und *Melolontha melolontha* (Feldmaikäfer) Larven stellen die ersten molekularen Diversitätsanalysen des Intestinaltraktes von Käferlarven überhaupt bzw. – zusammen mit einer Studie über Asseln (Kostanjsek et al., 2002) – die ersten umfassenden molekularen Untersuchungen des Intestinaltraktes anderer Boden-Arthropoden als Termiten dar. Mit der Inventarisierung der bakteriellen (277 Sequenzen) und archaeellen (98 Sequenzen) Diversität im Verdauungstrakt von Scarabaeidenlarven wurde ein wichtiger Datensatz geschaffen, der einerseits intensive Vergleiche mit dem gut untersuchten Termitensystem ermöglicht, andererseits aber auch eine breite Vergleichsgrundlage für jede weitere Diversitätsstudie an Invertebraten sein kann. Darüber hinaus eröffnet sich die Möglichkeit, über Vergleiche der aus Rosen- und Maikäferlarven

(und zukünftig vielleicht weiteren Scarabaeidenlarven) klonierten Sequenzen, nach Hinweisen für eine Co-Evolution von Scarabaeidenlarven und Vertretern ihrer Darmmikrobiota zu suchen, die für Termiten bereits gezeigt werden konnte (Lilburn et al., 1999).

Darmspezifität der Mikrobiota im Verdauungstrakt von Scarabaeidenlarven

Über vergleichende Untersuchungen der mikrobiellen Diversität im Futter der Larven und ihrer Haupt-Darmabschnitte, insbesondere durch Einsatz der T-RFLP-Technik, konnte gezeigt werden, dass sich die Darmflora von Rosenkäfer- und Maikäferlarven deutlich von der des gefressenen Futters unterscheidet. Wie die im Rahmen dieser Arbeit durchgeführten Untersuchungen an Regenwürmern zeigen, ist dies keineswegs selbstverständlich (s. Abb. 4.5). Die Mikroorganismen im Verdauungstrakt der Scarabaeidenlarven stammen offensichtlich zu einem Großteil nicht aus dem aufgenommenen Futter, so dass man von einer speziellen Funktion dieser Mikroorganismen auszugehen hat, die weit über die einer bloßen Nahrungsquelle für den Wirt hinausgeht. Aufgrund der Komplexität des Darmsystems Boden fressender Termiten wurde eine spezifische Darmflora für diese Tiere grundsätzlich in Frage gestellt (Bignell, 1994). Jüngste Untersuchungen an *Cubitermes*-Arten haben allerdings auch dort deutliche mikrobielle Diversitätsunterschiede zwischen Futterboden und verschiedenen Darmabschnitten aufgezeigt, so dass auch für Boden fressende Termiten eine spezifische Darmflora mittlerweile als gesichert gilt (Friedrich et al., 2001; Schmitt-Wagner et al., 2003b).

Zwei Ausnahmen sollen kurz erwähnt werden. Mesophile *Crenarchaeota*, die u.a. auch im Darm Boden fressender Termiten nachgewiesen werden konnten (Friedrich et al., 2001), wurden als einzige Archaeengruppe identifiziert, die nicht spezifisch für den Darm der Rosenkäferlarven war, sondern eindeutig aus dem Futterboden stammte (s. Kapitel 3.2). *Pantoea agglomerans*, ein ubiquitär verbreitetes *γ-Proteobacterium* (Gavini et al., 1989), wurde in Mittel- und Enddarm der Maikäferlarven nachgewiesen. Im Enddarm war es weniger häufig und auf die Lumenfraktion beschränkt, im Mitteldarm dagegen, dessen bakterielle Diversität z.T. extrem gering war, war es teilweise sehr dominant. T-RFLP-Analysen der bakteriellen Diversität von Futterwurzeln lieferte Hinweise, dass *P. agglomerans* über die Nahrung in den Darm gelangen könnte (s. Kapitel 3.4). Ob diesem Bakterium eine spezielle Funktion im Darm der Maikäferlarven zukommt, ist unklar. In Heuschrecken z.B., die *P. agglomerans* ebenfalls mit der Nahrung aufnehmen, ist es an der Synthese von Hormonen beteiligt (Dillon und Charnley,

2002; Dillon et al., 2000). Eine wichtige Voraussetzung, um eine spezifische Funktion von *P. agglomerans* für Maikäferlarven annehmen zu können, wäre eine permanente Assoziation beider Organismen, wie sie z.B. für Fransenflügler nachgewiesen werden konnte (de Vries et al., 2001). Dies konnte für Maikäferlarven allerdings nicht gezeigt werden.

Räumliche Verteilung der Mikrobiota zwischen Mittel- und Enddarm

Die räumliche Verteilung mikrobieller Populationen innerhalb der Verdauungstraktes, d.h. in den zwei Haupt-Darmabschnitten (Mittel- und Enddarm) von Scarabaeidenlarven, war ein Schwerpunkt dieser Arbeit. Die Analyse der physiko-chemischen Bedingungen zeigte bei beiden Scarabaeidenlarven deutliche Unterschiede zwischen Mittel- und Enddarm auf, die daher als zwei grundsätzlich verschiedene Habitate angesehen werden müssen (Tab. 4.1).

Tabelle 4.1: Vergleich einiger physiko-chemischer und mikrobiologischer Parameter der beiden Haupt-Darmabschnitte von Rosenkäfer- und Maikäferlarven (L2/L3-Stadien). Dom. = dominierende.

	Mitteldarm		**Enddarm**	
	Rosenkäfer	Maikäfer	Rosenkäfer	Maikäfer
max. pH	10,2	8,2	8,4	8,6
Redoxpotential [mV]	+75 bis –175	+340 bis +220	–25 bis –125	+75 bis 0
Dom. Fettsäure	Lactat, Acetat	Acetat	Acetat, Lactat	Acetat
O_2-Eindringtiefe	100 µm, Zentrum anoxisch			
Methanemission* [nmol g^{-1}FG h^{-1}]	keine	keine	30 / 120 - 360	23 / 51
Zelldichte [10^{10} Zellen g^{-1}FG]	0,89	0,32	4,0	1,5

* erster Wert: isolierte Enddärme, zweiter Wert: intakte Larven

Die unterschiedlichen physiko-chemischen Bedingungen spiegelten sich in unterschiedlichen Besiedlungsdichten mit Mikroorganismen sowie summarischen Parametern ihrer Aktivität (Fettsäurespektren, Methanemissionsraten) wider. Grundsätzlich war der als Gärkammer aus-

geprägte Enddarm dichter mit Mikroorganismen besiedelt als der Mitteldarm. Methanemission war stets auf den Enddarm beschränkt. Während bei den Maikäferlarven auch keine archaeellen 16S rRNA-Gene aus dem Mitteldarm amplifiziert werden konnten, wurden im Mitteldarm von Rosenkäferlarven 16S rRNA-Gene von methanogenen Vertretern der Familie *Methanobacteriaceae* detektiert. FISH-Zählungen (Kapitel 3.3) bestätigten aber die Vermutung, dass die absolute Anzahl methanogener *Archaea* hier sehr gering ist (< 0,1% aller Zellen). Der Mitteldarm von Scarabaeidenlarven stellt offensichtlich kein geeignetes Habitat für *Archaea* dar.

T-RFLP-Analysen der *Bacteria*-Gemeinschaft im Darm individueller Maikäferlarven (Kapitel 3.4) zeigten eindeutig, dass der Mitteldarm dieser Larven (trotz hoher Glucose-Konzentrationen und eines nur leicht alkalischen pH-Wertes!) keine stabile Bakterienpopulation beherbergt, sondern von starken Schwankungen der Diversität geprägt ist. Dies deutet an, dass Bakterien in diesem Darmabschnitt vermutlich ohne spezielle Funktion sind, außer der vielleicht, als Nahrungsquelle zu dienen. In diesem Zusammenhang sind die Ergebnisse der mit Rosenkäferlarven erzielten Diversitätsstudie (Kapitel 3.2), zumindest für den Mitteldarm, noch einmal kritisch zu hinterfragen. Der hier gewählte Weg, die Diversität aus gepoolten Darmproben zu bestimmen, um einen Überblick über die generelle Diversität zu bekommen, kann individuelle Unterschiede maskieren, die Hinweise auf die Funktion der untersuchten Darmabschnitte geben können. Systematische Untersuchungen an individuellen Rosenkäferlarven müssten zeigen, inwieweit die bakterielle Gemeinschaft (oder einzelne Gruppen) in diesem teilweise extrem alkalischen Kompartiment tatsächlich als stabil anzusehen ist. Die bisher durchgeführten FISH-Zählungen (Kapitel 3.3) zeigen, dass im Mitteldarm der Rosenkäferlarven (unabhängig vom Fixierungsmittel) ein deutlich geringerer Prozentsatz aller Zellen mit den allgemeinen *Bacteria*-Sonden detektiert wurde als im Enddarm. Dies könnte auf eine geringere Aktivität der Bakterien in diesem alkalischen Kompartiment zurückzuführen sein. Auch im extrem alkalischen P1-Kompartiment Boden fressender Termiten war der Anteil der Zellen, die mit den allgemeinen *Bacteria*-Sonden detektiert werden konnten, deutlich geringer als in den weniger alkalischen Kompartimenten (Schmitt-Wagner et al., 2003b).

Neben der bakteriellen und archaeellen Diversität wurde im Rahmen dieser Arbeit auch erstmals die Diversität pilzlicher Gemeinschaften in verschiedenen Abschnitten eines Arthropodendarms mit einer molekularen Fingerprint-Methode untersucht (Kapitel 3.3). Hefen und Trichomyceten sind z.B. als Symbionten des Intestinaltraktes von Arthropoden (z.B. Termi-

ten) bekannt (Misra, 1998; Prillinger et al., 1996). Auch wenn die Untersuchungen mit Scarabaeidenlarven erst am Anfang stehen, zeigt sich doch jetzt schon, dass die Zusammensetzung der Pilz-Gemeinschaften im Mittel- und Enddarm von Rosenkäferlarven deutlich verschieden ist. Auch in der Besiedlung mit Pilzen spiegelt sich also die Unterschiedlichkeit der beiden Darmabschnitte wider, die bereits aus der Analyse der *Bacteria*- und *Archaea*-Gemeinschaften deutlich wurde. Die deutlichen Unterschiede zwischen Mittel- und Enddarm sprechen gegen eine Bedeutung der Pilze als reine Futterquelle, wie sie z.B. für Regenwürmer angenommen wird (Bonkowski et al., 2000; Edwards und Bohlen, 1996), sondern deuten eine spezifische Funktion der Pilze, zumindest im Enddarm der Rosenkäferlarven an. Für Termiten wurde z.B. eine Beteiligung von Hefen am Polymerabbau postuliert (Schäfer et al., 1996).

Phylogenetische Diversität der Darmmikrobiota von Scarabaeidenlarven

Viele der in den Intestinaltrakten von Scarabaeidenlarven über ihre 16S rRNA-Gene detektierten Bakterien ließen sich phylogenetischen Gruppen zuordnen, die typisch für Verdauungstrakte sind (z.B. *Actinobacteria*, *Bacteroidetes*, *Clostridiales*, *Lactobacillales*, *Proteobacteria*). Im Gegensatz zu Holz fressenden Termiten wurden allerdings keine Spirochaeten detektiert, die dort bis zu 50% aller Prokaryonten ausmachen können (Hongoh et al., 2003; Paster et al., 1996). Das hohe Ausmaß bakterieller Diversität, das viele bislang nicht kultivierte Bakterienarten vermuten lässt, stimmte mit molekularen Untersuchungen anderer Intestinalsysteme gut überein (s.u.). Sowohl die Klone aus den Rosenkäfer- als auch aus den Maikäferlarven gruppierten vielfach mit Klonen, aber auch bereits isolierten Mikroorganismen, aus Intestinalsystemen anderer Insekten, z.B. Termiten (Hongoh et al., 2003; Schmitt-Wagner et al., 2003b), aber auch höherer Tiere wie Schweine (Leser et al., 2002) oder Rinder (Tajima et al., 1999; Whitford et al., 1998), einschließlich des Menschen (Suau et al., 1999). Auch diese Ergebnisse zeigen, dass es sich bei der Darmflora der Scarabaeidenlarven um eine spezifische Darmflora handelt. Die phylogenetische Analyse lässt dabei verschiedene Grade der Darmspezifität erahnen (Abb. 4.1). Es gab „Intestinal-Cluster“, in denen Maikäferklone mit Klonen aus Intestinaltrakten höherer Tiere gruppierten; diese Cluster könnten Mikroorganismen repräsentieren, die an generelle Eigenschaften von Intestinalsystemen adaptiert sind. „Insekten-Cluster“ wurden von Termiten-, Rosenkäferlarven- und Maikäferlarven-Klonen gebildet; sie könnten für Mikroorganismen stehen, die an die speziellen Eigenschaften von Insektendärmen angepasst sind.

tendärmen angepasst sind. Dementsprechend wären die Sequenzen aus „Maikäferlarven-Clustern“ Bakterien zuzuordnen, die speziell an diesen Wirt angepasst sind.

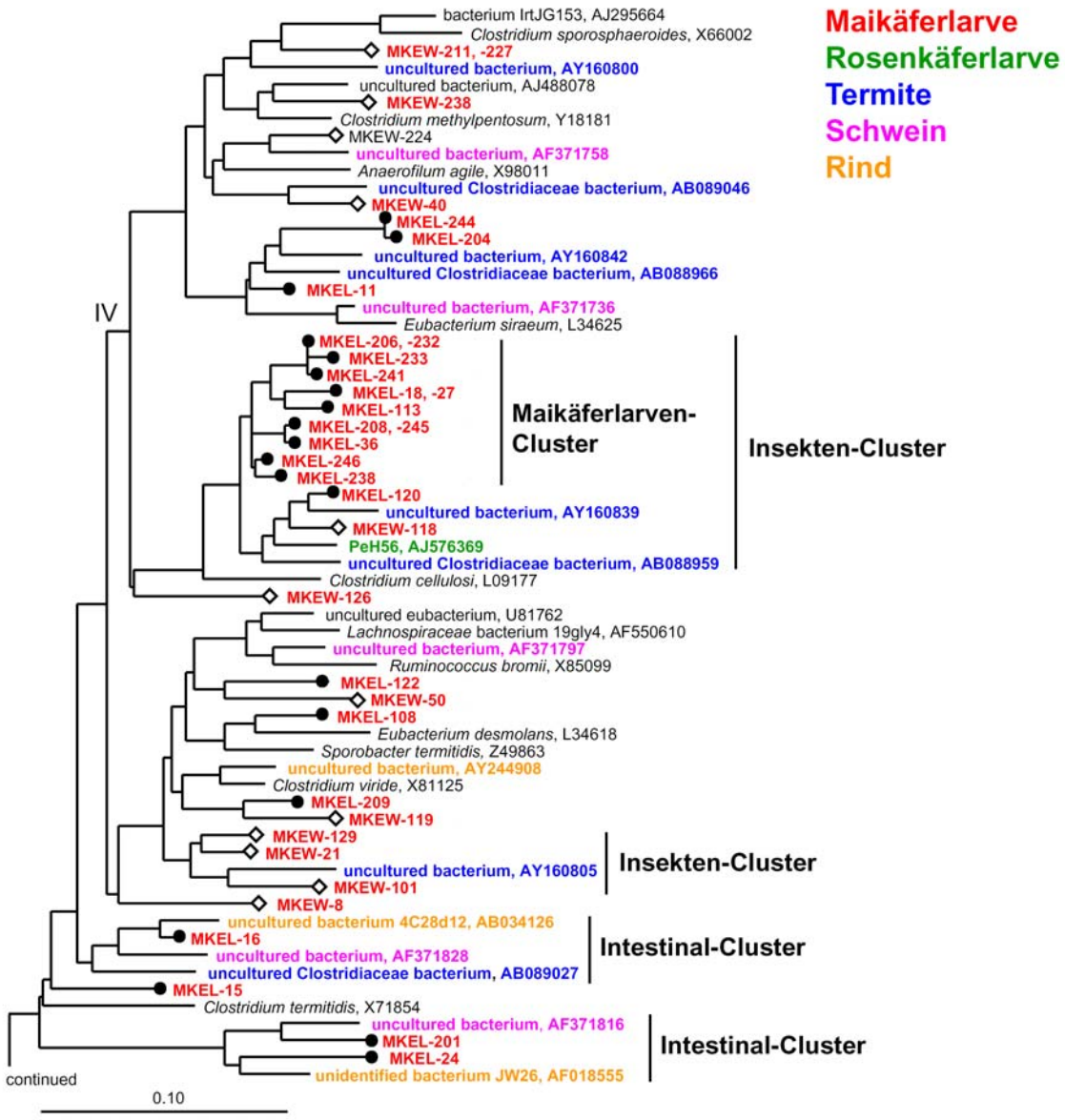

Abbildung 4.1: Ausschnitt aus dem in Kapitel 3.5 präsentierten phylogenetischen Baum, der die Position von Klonen aus dem Enddarm von Maikäferlarven mit Verwandtschaft zu den *Clostridiales* zeigt. Die Herkunft aller klonaler Sequenzen aus Intestinaltrakten ist farbig codiert. Der Maßstab zeigt 10% Sequenzunterschied an. Klone aus dem Darmlumen von Maikäferlarven sind mit Punkten, Darmwand-Klone mit Rauten gekennzeichnet. IV bezeichnet das Clostridien-Cluster IV sensu (Collins et al., 1994).

In einigen Fällen (Abb. 4.2) gruppierten Klone aus Rosen- und Maikäferlarven derart miteinander, dass man vielleicht von „Scarabaeiden-Clustern“ sprechen kann. Die Analyse weiterer

Scarabaeidenlarven müsste zeigen, ob dies gerechtfertig ist, und ob sich, ähnlich wie bei den Spirochaeten Holz fressender Termiten (Lilburn et al., 1999), Anzeichen für eine Co-Evolution der Scarabaeiden mit ihrer Intestinalflora finden lassen.

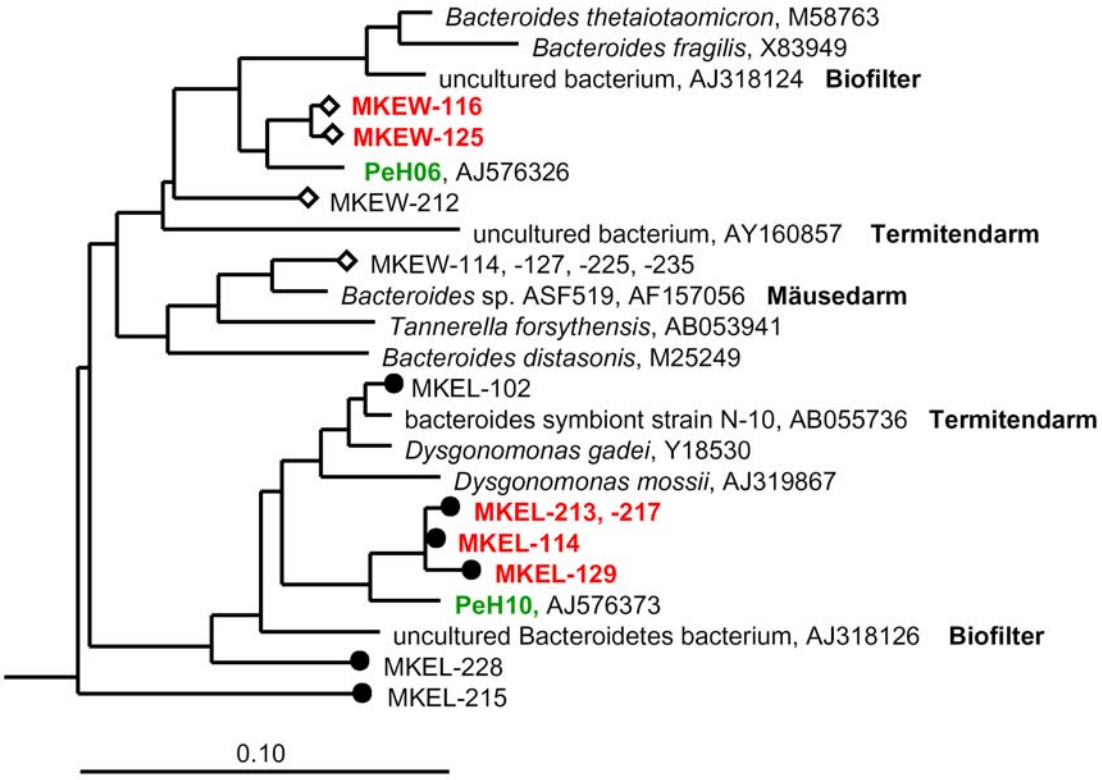

Abbildung 4.2: Ausschnitt aus dem in Kapitel 3.5 präsentierten phylogenetischen Baum, der die Position von Klonen aus dem Enddarm von Maikäferlarven mit Verwandtschaft zu den *Bacteroidetes* zeigt. Klone in zwei potentiellen „Scarabaeiden-Clustern" sind farbig codiert (rot: Maikäferlarven-Klone, grün: Rosenkäferlarven-Klone). Der Maßstab zeigt 10% Sequenzunterschied an. Klone aus dem Darmlumen von Maikäferlarven sind mit Punkten, Darmwand-Klone mit Rauten gekennzeichnet.

In diesem Zusammenhang ist auch erwähnenswert, dass in beiden Scarabaeidenlarven 16S rRNA-Gensequenzen mit enger Verwandtschaft zu *Turicibacter sanguinis*, einem strikt anaeroben, Lactat-bildenden Bakterium mit niedrigem G+C-Gehalt, gefunden wurden, das erstmals aus dem Blut eines Menschen mit akuter Blinddarmentzündung isoliert wurde (Bosshard et al., 2002). Ähnliche Sequenzen wurden auch im Darm von Mäusen, aber bisher noch nicht im Darm anderer Insekten gefunden.

Welche Funktionen die detektierten Bakterien im Einzelnen wahrnehmen, ist allein auf Grundlage von 16S rRNA-Gensequenzen nicht abzuschätzen, zumal viele Sequenzen nur entfernt verwandt zu denen bereits kultivierter Bakterien waren. Dass viele Klone aus beiden Käferlarven mit hydrolytischen und cellulolytischen Bakterien (z.B. *Actinobacteria*, *Clostridia*-

les, *Bacteroidetes*) und solchen mit ausgeprägtem Gärungsstoffwechsel (z.B. *Clostridiales*, *Lactobacillales*) gruppierten, zeigt aber eindeutig, dass ein Großteil der detektierten Bakterien vermutlich aktiv am Abbau organischer Substanz im Verdauungstrakt der Käferlarven beteiligt ist, worauf auch die in beiden Larven bestimmten hohen Konzentrationen an kurzkettigen Fettsäuren (v.a. Acetat und Lactat) hinweisen.

Die extrem hohen pH-Werte im Darm der Rosenkäferlarven (Kapitel 3.1; Tab. 4.1) zeigen, dass diese Tiere ihr Futter vermutlich einer alkalischen Extraktion unterziehen, ähnlich wie es Boden fressende Termiten tun (Kappler und Brune, 1999). Vermutlich verwerten auch sie eher die leichter verdaulichen Bestandteile (Peptide, pflanzliche Strukturpolysaccharide, event. mikrobielle Biomasse) ihrer Kost anstelle des polyaromatischen Anteils der Humine (Ji et al., 2000; Ji und Brune, 2001), entsprechende Untersuchungen werden z. Zt. durchgeführt (X. Li und A. Brune, unveröffentlicht). Hydrolytische Enzymaktivitäten (Proteasen, Cellulasen, Amylasen, Xylanasen) im Darm von Scarabaeidenlarven sind seit langem bekannt (Bauchop und Clarke, 1975; Biggs und McGregor, 1996; Cazemier et al., 1997b; Schlottke, 1945; Wagner et al., 2002; Werner, 1926), ihr genauer Ursprung (mikrobiell oder wirtseigen) ist aber größtenteils noch ungeklärt. Sechs Klone aus dem Mitteldarm der Rosenkäferlarven waren eng verwandt (> 98% Sequenzidentität) zu *Promicromonospora pachnodae*, einem hemicellulolytischen Actinobacterium, isoliert aus dem Enddarm von *Pachnoda marginata* Rosenkäferlarven (Cazemier et al., 2003). Zumindest ein Klon war entfernt verwandt zu einem alkaliphilen Bacillus, der eine β-1,3-Glucanase mit einem pH-Optimum von 9-10 ausscheidet (Nogi und Horikoshi, 1990). Ob Bakterien aber wirklich auch im extrem alkalischen Mitteldarm der Rosenkäferlarven an der Transformation organischer Substanz beteiligt sind oder ob diese Beteiligung erst im Enddarm zum Tragen kommt, wo viele Klone mit Verwandtschaft zu *Clostridiales* und *Bacteroidetes* detektiert wurden, ist noch ungeklärt.

Im Gegensatz zu den Rosenkäferlarven war der Mitteldarm der Maikäferlarven nur mäßig alkalisch (Kapitel 3.4; Tab. 4.1). Offensichtlich erfordert ihre Kost (Wurzeln) keine stark alkalischen pH-Werte, die nicht nur als Anpassung an Bodenfraß („alkalische Extraktion“), sondern auch an Tannin- bzw. Polyphenol-reiche Kost interpretiert werden, wo sie ein Ausfällen von Nahrungsproteinen bzw. Verdauungsenzymen verhindern sollen (Felton und Duffey, 1991; Martin et al., 1987). Studien an phytophagen *Costelytra zealandica* (*Melolonthinae*) Larven (Bauchop und Clarke, 1975; Bauchop und Clarke, 1977) kamen zu dem Schluss, dass diese v.a. die leicht verdaulichen Bestandteile von Wurzeln (z.B. Stärke) und weniger die Struktur-

polysaccharide verwerten. Da der Anteil leicht verdaulicher Kohlenhydrate in Wurzeln relativ gering ist (1-8% der Strukturpolysaccharide in Graswurzeln; (Steen und Larsson, 1986)), würde dies die starke Fraßaktivität von *C. zealandica* Larven erklären. Hohe Glucosekonzentrationen im Mitteldarm der Maikäferlarven deuten an, dass sie die leicht verdaulichen Bestandteile von Wurzeln wohl tatsächlich umsetzen, die Zusammensetzung der mikrobiellen Gemeinschaften im Enddarm zeigt aber eindeutig, dass sie auch das Potential haben sollten, pflanzliche Strukturpolysaccharide wie Cellulose zu verdauen. Gezielte Fütterungsversuche müssten dies zeigen. Im Gegensatz zu *C. zealandica* konnte zumindest im Enddarm von Maikäferlarven Carboxymethylcellulase-Aktivität nachgewiesen werden (Ricou, 1958).

FISH-Zählungen belegen, dass *Archaea*, deren Vorkommen nahezu auf den Enddarm der Scarbaeidenlarven beschränkt war, im Vergleich zu den Bakterien zahlenmäßig nur eine untergeordnete Rolle spielen (ca. 0,2 - 1,0% aller Bakterien). Im Vergleich zu den Bakterien war auch ihre Diversität deutlich geringer. Im Enddarm beider Käferlarven wurden Klone mit Verwandtschaft zu *Methanobrevibacter arboriphilus* (Miller, 2001), im Darm der Rosenkäferlarven zusätzlich Verwandte zu *Methanomicrococcus blatticola* (Sprenger et al., 2000) als methanogene *Archaea* identifiziert. Im Enddarm der Rosenkäferlarven kamen zudem Klone mit Verwandtschaft zu mesophilen *Thermoplasmales* vor, die ebenfalls in Termiten gefunden wurden (Friedrich et al., 2001; Shinzato et al., 1999). Alle Klone aus den Scarabaeidenlarven waren eng untereinander und mit Klonen aus der Boden fressenden Termite *Cubitermes orthognathus* verwandt. Die im Vergleich zu den phytophagen Maikäferlarven höheren Methanemissionsraten der humivoren Rosenkäferlarven stimmten mit höheren relativen Zellzahlen bei den Rosenkäferlarven überein. Diese Ergebnisse ähneln dem bei Termiten beobachteten Trend, dass Holz fressende Termiten weniger Methan emittieren und (relativ zu den Bakterien) weniger Methanogene beherbergen als Boden fressende Termiten (Brauman et al., 1992; Brauman et al., 2001).

Subkompartimentelle Topologie der Darmmikrobiota im Enddarm von Maikäferlarven

Die an Maikäferlarven durchgeführten topologischen Untersuchungen beschränkten sich nicht nur auf axiale Vergleiche mikrobieller Gemeinschaften in verschiedenen Darmabschnitten, sondern umfassten insbesondere radiale Vergleiche verschiedener Fraktionen (Darmwand und –lumen) eines Abschnittes (Kapitel 3.5). Solche Analysen haben bei Arthropoden noch abso-

luten Pioniercharakter und wurden auch bei Termiten (vermutlich wegen der geringen Größe) erst einmal durchgeführt (Berchtold et al., 1999). Für ein funktionelles Verständnis von Insektendärmen sind derartige Untersuchungen aber von großer Bedeutung, da diese aufgrund ihrer geringen Größe von steilen radialen Gradienten (z.B. O_2 und H_2) geprägt sind, die sich sowohl auf die Zusammensetzung der mikrobiellen Gemeinschaften (viele Aerotolerante) als auch auf deren Funktionen (Veränderung der Fermentationsbilanzen mit O_2 als Elektronenakzeptor, s. Kapitel 1.2) auswirken (Brune, 1998; Brune und Friedrich, 2000). Der Enddarm von Scarabaeidenlarven ist zwar ca. 100fach größer als der einer Termite, trotzdem hat auch er (im Vergleich z.B. zum Pansen von Rindern) noch ein hohes Oberfläche zu Volumenverhältnis. Geht man von einer maximalen O_2-Eindringtiefe von 100 µm aus (s. Tab. 4.1), so muss man ca. 10% des Enddarmvolumens einer Scarabaeidenlarve als mikrooxisch ansehen (Abb. 4.3). Sauerstoff stellt daher vermutlich auch im Scarabaeidendarm einen wichtigen Elektronenakzeptor dar.

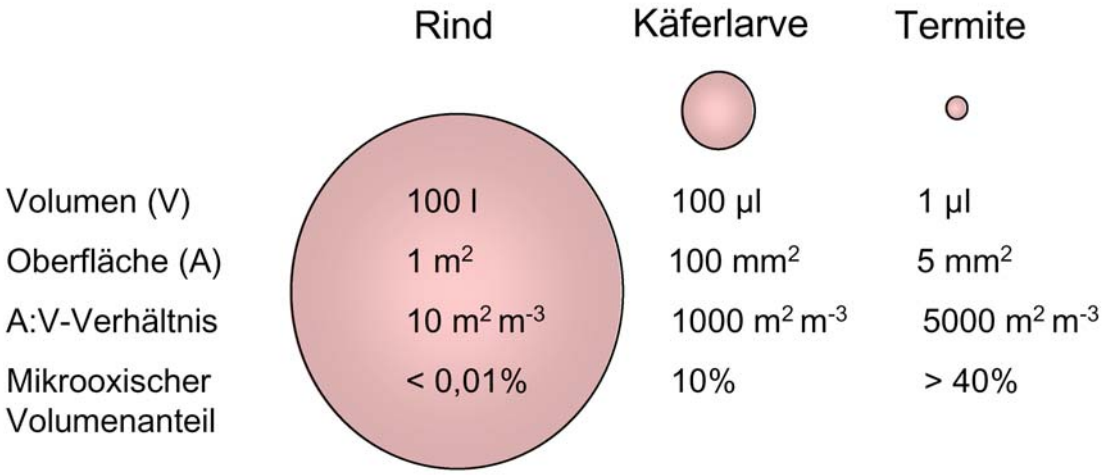

Abbildung 4.3: Vergleich des Oberfläche-zu-Volumenverhältnises des Pansens eines Rindes mit den Enddärmen einer Scarabaeidenlarve und einer Termite (idealisiert als Kugeln; Abb. nicht maßstabsgerecht) sowie des sich aus einer Eindringtiefe von 100 µm ergebenden mikrooxischen Volumenanteils (ergänzt nach (Brune, 1998)).

T-RFLP-Analysen bakterieller 16S rRNA-Gene zeigten, dass die bakterielle Gemeinschaft an der Enddarmwand verschiedener Maikäferlarven-Individuen mit statistischer Signifikanz ähnlicher zueinander ist als die *Bacteria*-Gemeinschaft an der Enddarmwand und im Lumen derselben Individuen (Kapitel 3.4). Vergleichbare Ergebnisse wurden erst kürzlich mit Boden fressenden Termiten erzielt: Es konnte gezeigt werden, dass die Bakteriengemeinschaften in

homologen Darmabschnitten verschiedener *Cubitermes*-Arten ähnlicher zueinander sind als die Gemeinschaften in benachbarten Abschnitten derselben *Cubitermes*-Art, in denen deutlich verschiedene physiko-chemische Bedingungen herrschen (Schmitt-Wagner et al., 2003a). Die im Rahmen dieser Arbeit an Maikäferlarven durchgeführte subkompartimentelle Analyse der räumlichen Verteilung bakterieller Gemeinschaften zeigte markante Unterschiede zwischen Enddarmwand und –lumen auf, die als Anpassungen an die spezifischen morphologischen (Chitinbäumchen als Aufwachsstrukturen) und vielleicht auch physiko-chemischen (Einstrom von O_2) Bedingungen an der Enddarmwand interpretiert werden müssen (Abb. 4.4).

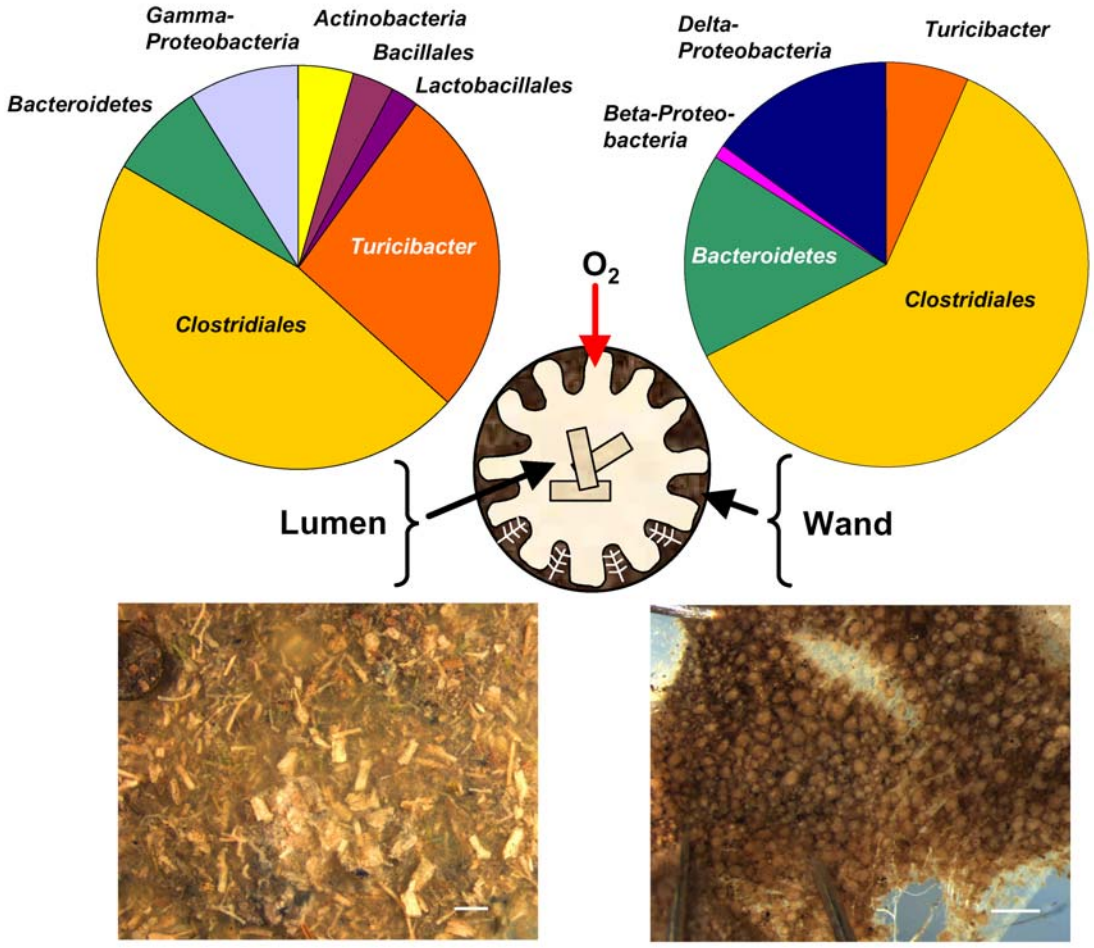

Abbildung 4.4: Zusammensetzung der bakteriellen Gemeinschaft im Lumen und an der Wand des Enddarms von Maikäferlarven. Dargestellt sind die relativen Häufigkeiten und phylogenetischen Zuordnungen von 16S rRNA-Genen in einer Lumen- (90 Klone) und einer Wand- (74 Klone) Klonbibliothek. An der Enddarmwand bilden die Mikroorganismen einen Knötchen-artigen Biofilm, der mehrere 100 µm dick werden kann (im schematischen Enddarm-Querschnitt dunkelbraun und überhöht dargestellt). Bäumchen-artige Strukturen aus Chitin (im Schema für einige Knötchen weiß dargestellt) dienen den Mikroorganismen als Aufwachshilfe. O_2 kann (in der gesamten Peripherie) bis zu 100 µm tief in den Darm eindringen (roter Pfeil). Im Darmlumen finden sich viele grobe Stückchen von Pflanzenmaterial. Maßstab in den Aufnahmen: 1 mm.

Actinobacteria, *Bacillales*, *Lactobacillales* und *γ-Proteobacteria* wurden ausschließlich im Darmlumen detektiert, *β-* und *δ-Proteobacteria* (zumindest mit den Klonbibliotheken) ausschließlich an der Enddarmwand. *Clostridiales* und *Bacteroidetes* kamen in beiden Fraktionen vor, allerdings zeigte die phylogenetische Analyse, dass bestimmte Untergruppen nur an der Enddarmwand, andere nur im Lumen vorkamen. So gruppierten z.B. alle *Bacteroidetes*-Verwandten des Lumens mit *Dysgonomonas*-Arten, die aus der Wandfraktion klonierten Arten dagegen mit *Bacteroides*- und *Cytophaga*-Arten (s. auch Abb. 4.2).

Der auffälligste Unterschied zwischen der Enddarmwand und –lumenfraktion der Maikäferlarven war allerdings die hohe Abundanz (ca. 10 – 15% aller *Bacteria*) *Desulfovibrio*-verwandter Bakterien an der Enddarmwand. Dieser Befund, der sowohl mit PCR-abhängigen als auch PCR-unabhängigen Methoden (FISH) bestätigt werden konnte, ist für Arthropoden bislang einmalig. Sulfat reduzierende Bakterien, insbesondere *Desulfovibrio*-Arten, sind zwar als Besiedler von Intestinaltrakten bekannt und wurden auch schon aus ihnen isoliert (Deplancke et al., 2000; Fröhlich et al., 1999; Loubinoux et al., 2000; Trinkerl et al., 1990), ihre räumliche Anordnung innerhalb des Intestinaltraktes war aber bislang nicht bekannt. Kuhnigk und Mitarbeiter (Kuhnigk et al., 1996) postulierten zwar schon 1996 einige Funktionen, die *Desulfovibrios* im Intestinaltrakt von Termiten wahrnehmen könnten und von denen die meisten auf einer Reduktion molekularen O_2 beruhen könnten (Oxidation von Lactat, Ethanol, Formiat, H_2, Sulfid), die dafür benötige räumliche Nähe zum Ort des O_2-Einstroms konnte aber erst hier zum ersten Mal zweifelsfrei bewiesen werden. Während die Fähigkeit, molekularen O_2 reduzieren zu können, für die Gattung *Desulfovibrio* schon seit längerem bekannt war (Cypionka, 2000; Kuhnigk et al., 1996), konnte dies erst kürzlich (Boga und Brune, 2003) auch für homoacetogene Bakterien aus dem Termitendarm (z.B. *Sporomusa aerivorans*) gezeigt werden. Selbst methanogene *Archaea* (*Methanobrevibacter* spp.), die an der Enddarmwand von Termiten nachgewiesen wurden, scheinen den Einstrom von O_2 dort tolerieren zu können (Leadbetter und Breznak, 1996).

Die hohen Sulfatkonzentrationen in Mittel- (fast 3 mM) und Enddarm (fast 1 mM) der Maikäferlarven (Kapitel 3.4) sprechen dafür, dass die detektierten Sulfatreduzierer neben O_2 auch Sulfat als Elektronenakzeptor verwenden könnten. Das Vorkommen von Sulfat in Mittel- und Enddarm spricht für eine Aufnahme mit dem Futter (Wurzeln, Boden), ob der Sulfatgehalt im Futter allerdings ausreicht, die sehr hohen Intestinalkonzentrationen zu erklären, ist bislang unklar. Zumindest für höhere Tiere sind auch endogene Sulfatquellen beschrieben worden

(Deplancke et al., 2000), z.B. in Form Sulfogruppen enthaltender Schleimstoffe (Sulfomucine; (Kent und Marsden, 1963)). Vergesellschaftet mit *Bacteroides fragilis*, der Sulfat aus Sulfomucinen freisetzen kann, konnte *Desulfovibrio desulfuricans* in-vitro mit Sulfomucinen als einziger C- und Energiequelle leben (Willis et al., 1996). In der Tat kommen im Maikäferdarm *Bacteroides*- und *Desulfovibrio*-Verwandte ausschließlich an der Enddarmwand miteinander vergesellschaftet vor (Kapitel 3.5).

FISH-Zählungen zeigten eindeutig und erstmals für Arthropoden auf einer quantitativen Basis, dass methanogene *Archaea* an der Enddarmwand der Maikäferlarven konzentriert sind (Kapitel 3.5). Dies erklärt, warum exogene H_2-Zufuhr schnell zu einer deutlichen Steigerung der Methanemission isolierter Enddärme führt. Eine ähnliche Steigerung konnte bei isolierten Rosenkäferlarven-Enddärmen auch durch Methanol und Formiat erzielt werden. Da Formiat auch in der Hämolymphe dieser Tiere vorkommt und isolierte Enddärme eine deutlich geringere Methanemission zeigen als intakte Larven, wurde ein Formiattransport aus dem Mitteldarm über die Hämolymphe postuliert, der die Methanogenese dort antreiben könnte (Kapitel 3.1). Die Steigerung der Methanemissionsraten durch externes Methanol stimmte gut mit dem Vorkommen von *Archaea* mit naher Verwandtschaft zu *Methanomicrococcus blatticola* im Enddarm der Rosenkäferlarven überein (Kapitel 3.2), einem obligat methlyotrophen Archaeon, das H_2 nur in Anwesenheit von Methanol oder Methylaminen als Elektronendonor benutzt (Sprenger et al., 2000).

4.2 Die Darmflora des Regenwurms – ein Vergleich mit anderen Invertebraten des Bodens

Regenwürmer stellen eine der wichtigsten Gruppen von Boden-Invertebraten dar, die, mit Boden-Mikroorganismen interagierend, zum Umsatz organischer Substanz in Böden beiträgt (Doube und Brown, 1998; Edwards und Fletcher, 1988). Die T-RFLP-Analyse bakterieller und archaeeller Gemeinschaften in Boden, Mitteldarm und Losung von *Lumbricus terrestris* Regenwürmern (Kapitel 3.7) ermöglichte aufgrund des integrativen Charakters der T-RFLP-Analyse erstmals einen Gesamtüberblick über die mikrobiellen Gemeinschaften im Verdauungstrakt dieses Regenwurms, im Vergleich zu den Gemeinschaften in Futter und Losung. Bislang existierte nur eine einzige umfassendere, kultivierungsunabhängige Untersuchung der Darmflora von Regenwürmern, in der *Archaea* allerdings unberücksichtigt blieben

(Schönholzer et al., 2002). In der einzigen Regenwurm-Studie, in der 16S rRNA-Gene (auch von *Archaea*) kloniert wurden (Furlong et al., 2002), wurden zwar Proben von Boden, Losung und Grabgängen, aber keine Darmproben analysiert.

Aufgrund der großen Ähnlichkeit der im Rahmen dieser Arbeit aufgenommenen T-RFLP-Profile von Futterboden, Mitteldarm und Losung der Regenwürmer kommt man zu dem Schluss, dass sowohl die Bakterien- als auch die Archaeengemeinschaften im Mitteldarm von *Lumbricus terrestris* zum größten Teil (wenn nicht völlig) aus dem Futterboden stammen, womit Ergebnisse früherer, kultivierungsabhängiger (Bassalik, 1913; Parle, 1963; Satchell, 1967) aber auch –unabhängiger Studien (Furlong et al., 2002) bestätigt werden.

Die Ähnlichkeit der mikrobiellen Gemeinschaften von Futter und Darm lässt sich quantitativ mit Hilfe von Morisita-Indizes (Dollhopf et al., 2001) beschreiben. Der Morisita-Index ist ein Maß für die Ähnlichkeit zweier Gemeinschaften und basiert auf der An- und Abwesenheit von Arten sowie deren relativen Häufigkeiten, im Falle des Vergleichs von T-RFLP-Profilen also dem Vorkommen und den relativen Häufigkeiten der T-RFs. Morisita-Werte liegen zwischen 0 und 1, wobei 1 für eine völlige (100%ige) Gleichheit der verglichenen Gemeinschaften steht. Die Untersuchungen an Regenwürmern wurden unter denselben PCR-T-RFLP-Bedingungen durchgeführt wie die Untersuchungen an Rosenkäferlarven (Kapitel 3.2) und eine erst kürzlich veröffentlichte Studie an Boden fressenden Termiten (Schmitt-Wagner et al., 2003a), in der ebenfalls bakterielle T-RFLP-Profile von Futterboden und Darm aufgenommen wurden. Hieraus ergab sich die Möglichkeit, die Ähnlichkeiten der bakteriellen Gemeinschaften von Futterboden und Darmflora verschiedener Boden fressender Invertebraten quantitativ miteinander zu vergleichen (Abb. 4.5).

Erst aus dem direkten Vergleich mit anderen Bodenfressern wird deutlich, wie ähnlich die Darmflora von *L. terrestris* der Bakteriengesellschaft des gefressenen Bodens ist und dass sich daraus der Schluss ergeben muss, dass sich im Mitteldarm dieses Regenwurms keine (dominante) spezifische Darmflora befindet. Eine Erklärung für das Fehlen einer spezifischen Darmflora ist die im Vergleich zu Scarabaeidenlarven und insbesondere Boden fressenden Termiten relativ einfache, schlauchförmige Darmmorphologie des Regenwurms, in der jegliche Kompartimentierung und insbesondere spezifische Aufwachsorgane für Mikroorganismen fehlen, wie sie z.B. als Chitinbäumchen (Hackstein und Stumm, 1994) bzw. -stacheln (Bignell et al., 1979) in Scarabaeidenlarven und Boden fressenden Termiten vorkommen.

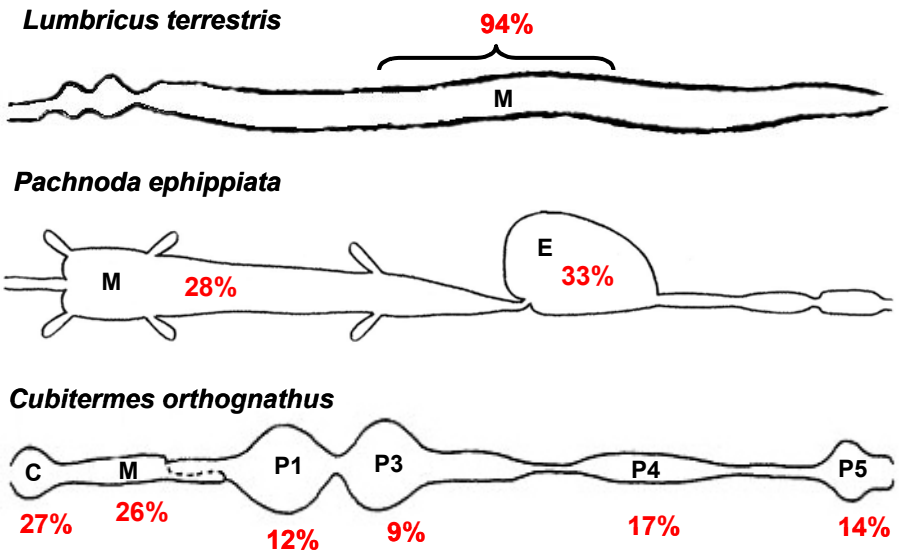

Abbildung 4.5: Prozentuale Ähnlichkeiten (Morisita-Indizes, berechnet nach T-RFLP-Profilen von 16S rRNA-Genen) zwischen der bakteriellen Gemeinschaft in verschiedenen Darmabschnitten Boden fressender Invertebraten (Regenwurm, Larve des Kongo-Rosenkäfers, Boden fressende Termite) und der bakteriellen Gemeinschaft im umgebenden Boden. C: Kropf, E: Enddarm, M: Mitteldarm, P: Paunch (= Enddarm). Abbildungen nicht maßstabsgerecht. Abbildungen des Regenwurm- und Termitendarms verändert nach (Horn et al., 2003) bzw. (Brune und Kühl, 1996). Werte für *C. orthognathus* aus (Schmitt-Wagner et al., 2003a).

Mit Hilfe multivariater Statistikmethoden konnten schließlich doch signifikante Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaften von Futterboden, Mitteldarm und Losung nachgewiesen werden, insbesondere, wenn die Würmer zusätzlich Buchenstreu zu fressen bekamen. Insgesamt ergab sich daher folgendes Bild: Die Darmflora von *L. terrestris* rekrutiert sich nahezu vollständig aus der mikrobiellen Gemeinschaft des gefressenen Bodens. Dass es doch signifikante Unterschiede zur Flora des umgebenden Bodens gibt, könnte folgende Gründe haben: a) gezielte Nahrungsaufnahme des Regenwurms an Stellen mit hoher mikrobieller Aktivität (Wolter und Scheu, 1999), b) ein Wechsel von aeroben zu anaeroben Bedingungen im Darm (Horn et al., 2003), der fakultativ Anaerobe bevorteilt (Karsten und Drake, 1995), c) schnelles Wachstum solcher Mikroorganismen (z.B. Pseudomonaden, (Furlong et al., 2002), die die günstigen Bedingungen im Darm (z.B. hoher Wasser- und Nährstoffgehalt, (Horn et al., 2003)) schnell ausnützen können, d) differentielles Lysieren bestimmter Gruppen von Mikroorganismen durch Verdauungsenzyme des Wurms (Edwards und Fletcher, 1988), e) Inhibition von Mikroorganismen durch Hemmstoffe, die von anderen Mikroorganismen ausgeschieden werden (Brown, 1995; Furlong et al., 2002).

Der Befund, dass die Unterschiede zwischen der Darmflora des Regenwurms und dem umgebenden Boden größer wurden, wenn die Würmer zusätzlich Buchenstreu zu fressen bekamen, erklärt sich zum einen mit der selektiven Aufnahme der Streu (und seiner Mikroorganismen) durch die Würmer. Zum anderen kann zusätzliche Streu im Futter einige der o.g. Faktoren verstärken, z.B. stellt sie einen zusätzlichen Nährstoffpool im Darm dar und erhöht vermutlich auch die Sekretion von Verdauungsenzymen. Außerdem verlangsamt sich die Darmpassage, wenn der Wurm frisst (Parle, 1963), und damit auch die Zeit, in der alle o.g. Faktoren auf die ingestierte Bodenflora einwirken können.
L. terrestris ist offensichtlich nicht auf eine spezifische Darmflora angewiesen. Die Mikroorganismen in seinem Darm stellen vermutlich eher Nahrung als Symbionten dar, die seine Verdauung unterstützen (Edwards und Fletcher, 1988). Weitere Untersuchungen müssten zeigen, inwieweit dies auch für Regenwürmer anderer Ernährungstypen (endogäische, hypogäische) gilt. Zumindest für *Eisenia foetida* (hypogäisch) wurde (mit kultivierungsabhängigen Methoden) eine spezifische Darmflora nachgewiesen (Toyota und Kimura, 2000). Ein erster Schritt um abzuklären, inwieweit sich die Aktivität bestimmter Gruppen von Mikroorganismen während der Darmpassage ändert, könnten auf rRNA basierende Diversitätsstudien sein. Hiermit ließe sich z.B. die Hypothese testen, dass endogäische, tropische Regenwürmer ingestierte Mikroorganismen über Schleimabsonderungen gezielt aktivieren, um sich deren großes katabolische Potential für ihre Verdauung zunutze zu machen (Barois, 1992; Lavelle et al., 1995; Trigo et al., 1999).

4.3 Die Bedeutung von pseudo-T-RFs für die T-RFLP-Analyse mikrobieller Gemeinschaften

Die T-RFLP-Analyse mikrobieller Gemeinschaften ist mittlerweile ein integraler Bestandteil des Methodenspektrums der molekularen mikrobiellen Ökologie. Als „Fingerprint“-Methode ermöglicht sie schnelle, übersichtsartige Vergleiche der Struktur mikrobieller Gemeinschaften in Abhängigkeit verschiedenster Faktoren (Ort, Zeit, abiotische Faktoren, biotische Faktoren etc.) mit hinreichender phylogenetischer Auflösung (Bruce und Hughes, 2000; Kitts, 2001).
Im Rahmen dieser Arbeit wurde entdeckt, dass sich T-RFLP-Profile PCR-amplifizierter 16S rRNA-Gene nicht nur aus T-RFs, sondern auch aus pseudo-T-RFs zusammensetzen, die nicht die terminalen, sondern längere (z.B. den zweiten oder dritten Schnittstellen einer Sequenz

zuzuordnende) Fragmente repräsentieren (Kapitel 3.8). Als Ursache für die Entstehung von pseudo-T-RFs wurden (partiell) einzelsträngige Amplikons identifiziert, die anscheinend unvermeidbar während der PCR entstehen und zur Bildung längerer Restriktionsfragmente führen, wenn die terminale Schnittstelle im einzelsträngigen Bereich liegt, wo sie von Restriktionsendonukleasen nicht geschnitten werden kann (Nishigaki et al., 1985).

Pseudo-T-RFs können, wenn sie unentdeckt bleiben, eine höhere Diversität in untersuchten Proben vortäuschen, als eigentlich vorliegt. Dies wird unmittelbar aus dem in Kapitel 3.8 und 3.9 präsentierten Profil der *Archaea*-Gemeinschaft im Mitteldarm der Rosenkäferlarven deutlich, in denen der dominante 165 bp-Peak keine dritte Gruppe von *Archaea* (neben *Methanobacteriaceae* und *Crenarchaeota*) repräsentiert, sondern ein pseudo-T-RF ist, das von Amplikons dieser beiden Gruppen verursacht wird. Eine Berücksichtigung dieses Artefaktes ist für eine sinnvolle Interpretation von T-RFLP-Profilen daher unbedingt nötig.

Im Rahmen der Diversitätsstudien an Scarabaeidenlarven wurde die T-RFLP-Analyse eingesetzt, um die über 16S rRNA-Gen Klonbibliotheken ermittelten Häufigkeiten phylogenetischer Gruppe zu kontrollieren, die aufgrund der beschränkten Anzahl analysierter Klone verzerrt seien können. Dabei wurde die Bildung von pseudo-T-RFs berücksichtigt, indem die Zuweisung einzelner T-RFs aus den Umweltprofilen auch auf Grundlage des in-vitro T-RF-Bildungsverhaltens einzelner Klone sowie der Veränderung der Umweltprofile nach Behandlung mit Mung bean-Nuklease und nicht nur auf Grundlage der aus den Klonsequenzen theoretisch (in-silico) abgeleiteten terminalen Fragmentlängen erfolgte. Der Erfolg einer solchen Vorgehensweise lässt sich aus einem Vergleich der mit und ohne Berücksichtigung dieses Artefaktes zuzuordnenden T-RFs in untersuchten Profilen ermessen: Im Falle der bakteriellen T-RFLP-Profile aus dem Enddarm von Maikäferlarven stieg der Anteil zuzuordnender T-RFs, gemessen als prozentualer Anteil der Peakhöhe aller zugeordneter Peaks zur Gesamt-Peakhöhe des jeweiligen Profils, von 71,2% (Lumenprofil) bzw. 65,0% (Wandprofil) auf 80,4 bzw. 80,6 % an. In der Rosenkäferlarven-Studie stiegen die Werte von 69,6% auf 88,1% (Enddarmprofil) bzw. von 77,5% auf 90,1% (Mitteldarmprofil) an. Diese Zahlen machen deutlich, dass durch die Berücksichtigung des Artefaktes der pseudo-T-RF-Bildung die Auswertung von T-RFLP-Profilen genauer wird. Der prozentuale Anteil von T-RFs, die phylogenetischen Gruppen aus einer Klonbibliothek zugeordnet werden können, ist ein Maß, wie gut die zugrunde liegende Klonbibliothek die Diversität im untersuchten System abgedeckt hat. Im Falle beider Diversitätsstudien mit Käferlarven konnte durch Berücksichtigung der pseu-

do-T-RF-Bildung eine deutliche Steigerung der zuzuordnenden T-RFs erzielt werden. Dabei wurde deutlich, dass die zugrunde liegenden Klonbibliotheken die Diversität in den untersuchten Proben tatsächlich viel besser widerspiegeln, als es ohne Berücksichtigung dieses Artefaktes den Anschein gehabt hätte.

Bei der T-RFLP-Studie mit Regenwürmern musste eine pseudo-T-RF-Bildung nicht berücksichtigt werden. Hier wurden ausschließlich gesamte Profile auf signifikante Unterschiede hin miteinander verglichen, ohne die einzelnen T-RFs bestimmten phylogenetischen Gruppen zuzuordnen. Die Bildung von pseudo-T-RFs hat sich als reproduzierbar und charakteristisch für die untersuchten Sequenzen erwiesen, d.h., wenn sich zwei Umweltprofile aufgrund des Auftretens von pseudo-T-RFs signifikant voneinander unterscheiden, so deutet dies auch auf signifikante Unterschiede in der Zusammensetzung der zugrunde liegenden mikrobiellen Gemeinschaft hin.

Die Ergebnisse einer Behandlung des Amplikonpools mit Klenow-Fagment (Kapitel 3.9), einer DNA-Polymerase die u.a. zum Auffüllen überhängender 5´-Enden verwendet wird, zeigen, dass sich hierdurch partiell einzelsträngige Amplikons wieder in doppelsträngige überführen lassen, die für Restriktionsenzyme direkt zugänglich sind. Der große Vorteil gegenüber einer Mung bean-Nuklease-Behandlung besteht darin, dass sich aus den so erzeugten Profilen die relativen Häufigkeiten der den jeweiligen T-RFs zuzuordnenden phylogenetischen Gruppen direkt abschätzen lassen, da die pseudo-T-RFs nicht einfach verdaut, sondern in „echte“ T-RFs überführt werden.

Auf Grundlage aller bisher erzielter Ergebnisse zur Bildung von pseudo-T-RFs können abschließend folgende allgemeine Empfehlungen zur Berücksichtigung dieses PCR-Artefaktes bei der T-RFLP-Analyse mikrobieller Gemeinschaften gemacht werden:

- PCR-Amplifikation mit möglichst geringer Zyklenzahl, da die pseudo-T-RF-Bildung linear mit der Zyklenzahl korreliert. Eine Empfehlung, die grundsätzlich für alle PCR-abhängigen Methoden gilt (Osborn et al., 2000; Polz und Cavanaugh, 1998; Suzuki et al., 1998; Suzuki und Giovannoni, 1996).
- Bei der Wahl des Restriktionsenzyms ist zu beachten, dass das Enzym, das experimentell Profile mit den meisten T-RFs liefert, nicht unbedingt das Enzym ist, welches die zugrunde liegende Diversität am besten auflöst, da es sich hierbei auch um pseudo-T-RFs handeln kann. Enzyme, die bei niedrigerer Temperatur (37°C) arbeiten sind zu bevorzugen,

da Sekundärstrukturen, die eine pseudo-T-RF-Bildung verhindern können, bei höheren Verdautemperaturen instabil werden (Kapitel 3.8). Kürzlich wurde, auf in-silico Untersuchungen zur Lage von Restriktionsschnittstellen in 16S rRNA-Genen fußend, *BstU*I als Restriktionsenzym empfohlen, das bakterielle Gemeinschaften mit hoher Diversität am besten aufzulösen vermag (Engebretson und Moyer, 2003). Allerdings arbeitet dieses Enzym bei 60°C und zeigte in Experimenten mit klonalen Amplikons die stärkste Tendenz zur pseudo-T-RF-Bildung. Sofern eine genügende Auflösung der untersuchten Sequenzen gewährleistet ist, ist *Taq*I als Restriktionsenzym zu empfehlen, da es (aus bislang unbekannten Gründen) nahezu keine pseudo-T-RF-Bildung zeigt.

- Sollen T-RFs aus Umweltprofilen zugrunde liegenden phylogenetischen Gruppen zugeordnet werden, ist die parallele Errichtung einer Klonbibliothek zu empfehlen. Bei der Zuweisung der T-RFs sollte stets das in-vitro T-RF-Bildungsverhalten der Klone sowie die Veränderung der Umweltprofile nach Mung bean-Nuklease- und/oder Klenow-Fragment-Behandlung berücksichtigt werden. Eine Abschätzung relativer Amplikonhäufigkeiten anhand der Höhe und/oder Fläche einzelner T-RFs ist nur in unbehandelten oder mit Klenow-Fragment behandelten Profilen möglich.
- Eine Zuweisung von T-RFs auf Grundlage einer Suche nach Sequenzen mit passenden Restriktionsschnittstellen in öffentlichen Datenbanken, die zunehmend propagiert wird (z.B. (Kent et al., 2003)), ist als kritisch einzustufen, insbesondere wenn diese T-RFs zuvor nicht auf Echtheit geprüft wurden, z.B. über einen Mung bean-Nuklease-Verdau.

Klärungsbedarf besteht noch hinsichtlich des Reaktionsmechanismus, der partiell einzelsträngige Amplikons während der PCR entstehen lässt. Außerdem ist bislang nicht bekannt, ob und inwieweit andere PCR-abhängige Methoden der mikrobiellen Ökologie (z.B. DGGE oder Klonierungsschritte) durch partiell einzelsträngige Amplikons beeinflusst werden. Ein erster Schritt zur Abschätzung der generellen Bedeutung dieses PCR-Artefaktes wäre, auch bei anderen Methoden den Einfluss einer Behandlung des Amplikonpools mit Mung bean-Nuklease und/oder Klenow-Fragment zu untersuchen.

4.4 Ausblick: Integration von Struktur und Funktion mikrobieller Gemeinschaften im Intestinaltrakt von Scarabaeidenlarven

Die im Rahmen dieser Arbeit erzielten Ergebnisse zur Struktur und räumlichen Verteilung der mikrobiellen Gemeinschaften im Intestinaltrakt von Scarabaeidenlarven stellen eine wichtige Grundlage dar, um bei zukünftigen Untersuchungen die Funktion der Mikroorganismen bei der Transformation organischer Substanz stärker in den Mittelpunkt zu stellen.

Zunächst sollen aber die laufenden PCR-unabhängigen Untersuchungen (FISH-Zählungen) zur Struktur und räumlichen Verteilung der Mikroorganismen im Darm der Rosenkäferlarven abgeschlossen werden. Auf den Ergebnissen mit Maikäferlarven aufbauend, sollte insbesondere die Besiedlung des extrem alkalischen Mitteldarmabschnittes der Rosenkäferlarven mit individuellen Larven auf seine Stabilität hin überprüft werden. Danach könnten auch bei diesen Larven, in ähnlicher Weise wie für die Maikäferlarven bereits geschehen, subkompartimentelle Abschnitte des Mitteldarms (Wand, Lumen, peritrophe Membran, verschiedene axiale Abschnitte entlang des pH-Gradienten) oder Enddarms (Wand, Lumen, einzelne Chitinbäumchen mit anhaftendem Biofilm) auf ihre mikrobielle Besiedlung hin untersucht und zu den dort herrschenden physiko-chemischen Bedingungen in Beziehung gesetzt werden. Die deutlichen Unterschiede in der Besiedlung der beiden Rosenkäfer-Darmabschnitte mit Pilzen machen deutlich, dass für eine tieferes Verständnis des Intestinaltraktes der Scarabaeidenlarven neben Bakterien und Archaea auch weitere Gruppen von Mikroorganismen (Pilze, aber auch Protozoen, Nematoden) in die Untersuchungen mit einbezogen werden könnten.

Untersuchungen zur in-situ Funktion von Mikroorganismen müssten sich an Ergebnissen von Fütterungsversuchen (z.B. mit ^{14}C-markierten Substraten wie Cellulose, Peptidoglykan, Chitin etc.) orientieren, die Aufschluss darüber erlauben, welche Stoffklassen mit welchen Raten im Scarabaeidendarm umgesetzt werden. Wichtige Folgeschritte wären Isolation und Kultivierung von Mikroorganismen, z.B. der *Desulfovibrio*-Verwandten an der Enddarmwand der Maikäferlarven, um deren tatsächliche physiologische Eigenschaften zu charakterisieren und daraus potentielle Funktionen im Darm ableiten zu können. Phylogenetische Untersuchungen von 16S rRNA- oder funktionellen Markergenen (z.B. *apsA*) aus anderen Feldmaikäfer-, Waldmaikäfer- oder Scarabaeidenpopulationen würden zeigen, wie spezifisch die Assoziation von *Desulfovibrios* mit Maikäfern bzw. Scarabaeiden ist und ob es Anzeichen für eine Co-Evolution gibt. Zu einem tieferen Verständnis der Funktion des bakteriellen Biofilms an der Enddarmwand der Maikäferlarven (und anderer Epithelien im Scarabaeidendarm) würde eine

Untersuchung der dreidimensionalen Struktur dieses Biofilms, d.h. der relativen Lage verschiedener funktioneller Gruppen zueinander (z.B. *Bacteroides* und *Desulfovibrio*) und zur Darmwand (als Quelle von Sauerstoff) unter Anwendung konfokaler Laser-Scanning-Mikroskopie beitragen, mit der in Ansätzen schon begonnen wurde (Kapitel 3.6). Um insbesondere die Funktion der Sulfatreduzierer näher zu beleuchten, sollte auch eine detaillierte Untersuchung des Schwefelpools und der verschiedenen im Darm und Futter der Maikäferlarven vorkommenden Schwefelspezies durchgeführt werden.

Eine vielversprechende Möglichkeit, die Beteiligung einzelner funktioneller Gruppen am C-Fluss in Insektendärmen gezielt zu untersuchen, ist die stabile Isotopenbeprobung von Nukleinsäuren (DNA, RNA) aktiver mikrobieller Populationen (Radajewski et al., 2000; Radajewski et al., 2003). Hierzu könnten zunächst Darmhomogenate mit ^{13}C-markierten Metaboliten inkubiert werden, deren Auswahl sich an den mit ^{14}C-markierten Monomeren (Glucose, Aminosäuren) und Gärprodukten (Lactat, Acetat, Propionat, Formiat) identifizierten mikrobiellen Aktivitäten polymerabbauender und gärender Mikroorganismen orientieren müsste. Nach der Inkubation können aktive Populationen nach Dichtegradientenauftrennung der Nukleinsäuren und (RT)-PCR-Amplifikation mittels T-RFLP-Fingerprinting Techniken bzw. 16S rRNA-Gen Klonbibliotheken im Vergleich zu den isotopisch leichteren Nukleinsäure-Fraktionen nachgewiesen werden (Lüders et al., 2003a; Lüders et al., 2003b). Weitergehende Untersuchungen könnten Mikroinjektionen ^{13}C-markierter Metabolite in isolierte, aber ansonsten intakte Darmabschnitte umfassen, bei denen die charakteristischen physikochemischen Bedingungen im Darm erhalten bleiben (Tholen und Brune, 2000). Schließlich kämen auch Fütterungsversuche mit ^{13}C-markierten Polymeren (Cellulose, Proteine, Peptidoglykan) in Betracht, um die am Umsatz dieser Stoffklassen beteiligten Mikroorganismen direkt zu identifizieren.

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Erklärung

Ich versichere, dass ich meine Dissertation

„Struktur und räumliche Verteilung mikrobieller Gemeinschaften im Verdauungstrakt ausgewählter Boden-Invertebraten“

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner anderen als der von mit ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, im November 2003